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| (71) Applicant (for all designated States except US): ELI LILLY AND COMPANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US). | | | |
| (72) Inventors; and (75) Inventors/Applicants (for US only): BRADER, Mark, Lawrence [NZ/US]; 5858 Forest Lane, Indianapolis, IN 46220 (US). HUGHES, Benjamin, Lee [US/US]; 402 Blue Ridge Road, Indianapolis, IN 46208 (US). LI, Shun [US/US]; 7620 Lancaster Drive, Indianapolis, IN 46268 (US). NG, Kingman [CN/US]; 10745 Putnam Place, Carmel, IN 46032 (US). SUKUMAR, Muppalla [IN/US]; 12604 Bent Oak Lane, Indianapolis, IN 46236 (US). WOLFF, Ronald, Keith [US/US]; 12329 Brookshire Parkway, Carmel, IN 46033 (US). | | | |
| (74) Agents: WALSH, Andrea, C. et al.; Lilly Corporate Center, Indianapolis, IN 46285 (US). | | | |

(54) Title: INSULIN CRYSTALS FOR PULMONARY ADMINISTRATION

(57) Abstract

The invention relates to crystals comprising derivatized insulin or derivatized insulin analog, to processes for preparing such crystals, and to methods of treating diabetes comprising administering the crystals to a patient in need thereof to control blood glucose. Crystals having a desired mean size are obtained. The mean crystal size may be controlled so that the crystals may be administered advantageously by inhalation through the mouth, deposited in the deep lung, and absorbed there over a protracted period of time, to provide control of blood glucose between meals and overnight.

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INSULIN CRYSTALS FOR PULMONARY ADMINISTRATION

Cross Reference

This application claims the benefit of U.S. 5 Provisional Application No. 60/131,170 filed on April 27, 1999, said application of which is entirely incorporated herein by reference.

Background of the Invention

10 1. Field of the Invention. The invention is in the field of human medicine. More particularly, the invention is in the field of the treatment of diabetes and hyperglycemia.

15 2. Description of Related Art. It has long been a goal of insulin therapy to mimic the pattern of endogenous insulin secretion in normal individuals. The daily physiological demand for insulin fluctuates and can be separated into two phases: (a) the absorptive phase requiring a pulse of insulin to dispose of the meal-related 20 blood glucose surge, and (b) the post-absorptive phase requiring a sustained delivery of insulin to regulate hepatic glucose output for maintaining optimal fasting blood glucose. Accordingly, effective therapy for people with diabetes generally involves the combined use of two types of 25 exogenous insulin formulations: a fast-acting meal time insulin provided by bolus injections and a long-acting, so-called, basal insulin, administered by injection once or twice daily to control blood glucose levels between meals.

Conventional insulin therapy involves only two 30 injections per day. More intensive insulin therapy involving three or more injections of insulin each day results in reduction of complications, as demonstrated in the Diabetes Control and Complications Trial (DCCT) study.

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Unfortunately, many diabetics are unwilling to undertake intensive therapy due to the discomfort associated with the many injections required to maintain close control of glucose levels. A non-injectable form of insulin is 5 desirable for increasing patient compliance with intensive insulin therapy and lowering their risk of complications.

Many investigators have studied non-injectable forms of insulin, such as oral, rectal, transdermal, and nasal routes. So far, these types of administration have 10 not been effective due to poor insulin absorption, low serum insulin concentration, irritation at the site of delivery, or lack of significant decrease in serum glucose levels.

Another well-studied route for administering non-injectable insulin is by the lung. Due to its relatively 15 small molecular weight (5,800 daltons) insulin seems to be an ideal candidate for administration through the lungs. Administration of insulin by inhalation, and its absorption through the lung was first reported in 1925.

However, after administration by inhalation, 20 small-sized proteins like insulin are absorbed rapidly from the lung due to the very large surface area and relatively porous membrane of the lung. The plasma concentration peaks quickly, but also decreases quickly. These pharmacokinetics may be suited for controlling blood glucose during the 25 absorptive phase, but are completely unsuited during the post-absorptive phase. Therefore, means for administration of long-acting insulin by inhalation remains a challenge.

The following are reviews of the inhalation of insulin and other proteins: "Aerosol Insulin- A Brief 30 Review", Patton, J. S. and Platz, R. M., in *Respiratory Drug Delivery IV*, P. Byron, Ed., Interpharm Press (1994); "Delivery of Biotherapeutics by Inhalation Aerosol", Niven, R., *Crit. Rev. in Therapeutic Drug Carrier Systems* 12:151-

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231 (1995); and "Drug Delivery via the Respiratory Tract",
Byron, P. R. and Patton, J. S., *J. Aerosol Medicine* 7:49-75
(1994).

5 The efficiency of delivery and deposition of
particles onto the surface of the deep lung, i.e. to the
alveoli, is strongly related to the mass median aerodynamic
diameter (MMAD) of the particles. The optimal MMAD is about
2-3 microns. Above and below this range, less material will
be deposited onto the alveolar surface.

10 Phagocytosis of insoluble or slowly dissolving
particles is the most significant clearance mechanism in the
deep lung [Rudt, S. H., et al., *J. Controlled Release*
25:123-138 (1993); Tabata, Y., et al., *J. Biomedical
Material Res.* 22:837-842 (1988); Wang, J. A., et al., *In:
Respiratory Drug Delivery*. Buffalo Grove, IL; Interpharm
1997, vol. VI, pp. 187-192; Edwards, D. A., et al., *Science*
276:1868-1871 (1997); Gehr, P. M., *Microsc. Res. Tech.*
26:423-436 (1993)]. Once deposited on the alveolar surface,
particles having actual dimensions in the range of about 1
20 to about 10 microns may be phagocytosed by lung macrophages.
It is known that very large particles, such as those having
actual diameters above about 10 microns, are not efficiently
phagocytosed by lung macrophages. Small particles with
actual dimensions in the nanometer size range, likely also
25 escape macrophage ingestion.

Unfortunately, particles having optimal properties
for delivery and deposition often have actual particle
dimensions that fall in the range within which macrophage
attack is expected.

30 Edwards, et al. describe protracted release of
insulin from large, porous particles administered into the
deep lung by inhalation [*J. Appl. Physiol.* 85:379-385
(1998); *Science* 276:1868-1871 (1997)]. The persistence of

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the administered particles was attributed to the large actual size of the particles (greater than 10 microns), while their high rate of deposition into the deep lung was attributed to the low density of the particles which 5 resulted in relatively small aerodynamic size (approximately 3-4 micrometers MMAD) despite their large physical size. These particles comprise insulin encapsulated in a biodegradable copolymer (poly-lactic-co-glycolic acid). Slow degradation of the copolymer releases insulin over a 10 period of at least four days. These publications demonstrate that a depot effect in the lung is a feasible mechanism for producing a sustained delivery of insulin.

Crystallization of insulin, such as with NPH-insulin and the various Lente products, is a well-known 15 means to provide extended control of blood glucose in people with diabetes. Insulin crystals have uniformly been administered by parenteral routes, usually subcutaneously. Acceptably high bioavailability of insulin-containing crystals when delivered to the deep lung by inhalation has 20 not been achieved. This is likely due to the complex protective mechanisms involving the biology, immunology, and chemistry of the lung surface relating to clearing of air-borne particles, especially microbes and aerosols containing microbes, which have particle qualities that permit their 25 evasion of upper respiratory entrapment mechanisms.

Hughes, B. L., et al. [PCT/US98/23040, filed 29 October 1998] described pharmaceutical compositions and methods of administering fatty acid-acylated insulin and insulin analogs by inhalation to treat diabetes. The 30 compositions were solutions or powders of amorphous materials, but not crystals. Hughes, et al. demonstrated that derivatized insulins, B28-Nε-myristoyl-LysB28, ProB29-human insulin analog and B29-Nε-palmitoyl-human insulin,

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were absorbed in an amount effective to reduce glucose levels. The pharmacokinetics of the fatty acid-acylated insulins administered via the lung was protracted compared with non-acylated insulin, but the length of protraction was 5 shorter than that of NPH-insulin delivered subcutaneously.

Havelund, S. [WO98/42749, published 1 October 1998] describes zinc-free crystal powders of insulin, insulin analogs, and insulin derivatives that are claimed to be resistant to aggregation and clumping, and allegedly 10 suitable for administration by inhalation.

Whittingham, J. L., et al. [Biochemistry 36:2826-2831 (1997)] produced very large crystals comprised of B29- $\text{N}\epsilon$ -tetradecanoyl-des(B30)-human insulin analog and zinc for structural studies by X-ray crystallography. These crystals 15 were much too large to expect efficient deposition in the deep lung when administered by inhalation. These crystals would have to have been milled to produce material of suitable particle size to achieve efficient deposition when administered by inhalation.

Brader, M. [U.S. Patent Application No. 09/177685, filed 22 October 1998; PCT/US98/22434; European Patent Publication No. 0911035, published 28 April 1999, herein 20 Brader I] and Brader, M. [U.S. Patent Application No. 09/217275, filed 21 December 1998; PCT/US98/27299, herein Brader II] describe microcrystals comprising divalent metal 25 cations together with derivatized proteins, including derivatized insulin and derivatized insulin analogs, processes for making the crystals, and methods for administering them to treat diabetes. The crystals are rod-shaped, and have the size of rod-shaped commercial NPH- 30 insulin crystals, which is about 5 microns in length. Such crystals are thought to be too large to obtain optimal

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deposition in the deep lung when administered by inhalation through the mouth.

Brader II lays out a long list of parameters that were thought to affect crystallization rate and size, and 5 among them were mentioned temperature and the concentration of competing compounds, such as citrate. Brader II does not however, specify the relationship between crystal size and temperature, and the effect of competing compound on size is only inferred from its likely effect in slowing down the 10 rate of crystallization. Furthermore, Brader II does not mention the chloride anion among the many parameters thought to influence crystal size of derivatized proteins.

Summary of the Invention

The invention includes a stable population of 15 crystals comprised of a derivatized insulin or a derivatized insulin analog and a divalent metal cation, characterized in that the crystals have a mean diameter in the range of 1-3 microns.

More specifically, the invention is crystals 20 having a uni-modal, symmetric particle distribution, comprising:

- a) derivatized insulin of the formula B29-NE-X-
human insulin, wherein X is selected from
the group consisting of butyryl, pentanoyl,
hexanoyl, heptanoyl, octanoyl, nonanyl, and
decanoyl;
- b) zinc;
- c) a phenolic preservative; and
- d) protamine;

30 characterized in that the volume mean spherical equivalent diameter is from 1 microns to 3 microns.

The invention includes a stable population of co-crystals comprised of an insulin or an insulin analog and a derivatized insulin or a derivatized insulin analog, characterized in that the co-crystals have a mean diameter 5 in the range of 1-3 microns. Thus, more specifically, the invention also includes co-crystals having a uni-modal, symmetric particle distribution, comprising insulin and:

- a) derivatized insulin of the formula B29-NE-X-
10 human insulin, wherein X is selected from the group consisting of butyryl, pentanoyl, hexanoyl, heptanoyl, octanoyl, nonanyl, and decanoyl;
- b) zinc;
- c) a phenolic preservative; and
- 15 d) protamine;

characterized in that the volume mean spherical equivalent diameter is from 1 microns to 3 microns, wherein the wherein the proportion of derivatized insulin relative to total protein is at least 50%.

20 The invention also includes pharmaceutical compositions comprising crystals or co-crystals together with one or more pharmaceutically acceptable excipients carriers, or with an aqueous solvent in which the crystals are stable. The pharmaceutical composition may be for 25 parenteral administration, or more preferably, for administration by inhalation through the mouth of the patient for deposition in the deep lung (the alveolae).

For parenteral pharmaceutical compositions, the crystals are suspended in a pharmaceutically acceptable 30 aqueous solvent, comprising optionally an isotonicity agent, a buffer, a preservative, and insulin or an insulin analog. For pharmaceutical compositions for pulmonary administration, the crystals may be in the form of a dry

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powder (for delivery by a dry powder inhaler for example), optionally together with other dry excipients, dry powders of insulin or an insulin analog, and carrier particles. The crystals may also be formulated for administration by 5 inhalation through the mouth of the patient in a liquid form by suspending them in a pharmaceutically acceptable aqueous solvent, comprising optionally insulin or an insulin analog.

The invention provides a process for preparing crystals and co-crystals of a size that increases the 10 efficiency of their deposition in the deep lung when administered by inhalation through the mouth. The process involves preparing a suspension having neutral pH, in the absence of citrate, by carrying out steps a) - f) in any order, provided that step f) follows step a), and provided 15 that if step f) precedes step e), then steps b) and c) precede step f):

- a) dissolving a derivatized insulin in an aqueous solvent at acidic pH;
- b) adding a phenolic preservative;
- 20 c) adding zinc;
- d) adding chloride anion to a final concentration of from about 100 mM to about 150 mM chloride anion above that introduced by pH adjustment;
- e) adding protamine;
- 25 f) adjusting to a neutral pH;

and then holding the temperature of the neutral pH suspension between about 25°C and about 37°C for between 12 hours and about 96 hours.

30 The invention also encompasses the use of crystals in the manufacture of a medicament for the treatment of diabetes or hyperglycemia by inhalation, which treatment comprises administering to a patient in need thereof an

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effective amount of the medicament using an inhalation device, such that the medicament is deposited in the lungs of the patient.

The invention also encompasses the use of co-crystals in the manufacture of a medicament for the treatment of diabetes or hyperglycemia by inhalation, which treatment comprises administering to a patient in need thereof an effective amount of the medicament using an inhalation device, such that the medicament is deposited in the lungs of the patient.

The present invention also provides a method for administering crystals or co-crystals by inhalation.

The invention also provides crystals and co-crystals that are advantageously more physically stable and easier to resuspend than crystals and co-crystals produced by the methods described by Brader I and Brader II. The process for making crystals and co-crystals with improved properties involves preparing a suspension having neutral pH, in the absence of citrate, by carrying out steps a) - g) in any order, provided that step g) follows step a), and provided that if step g) precedes step f), then steps b) and c) precede step g):

- a) dissolving a derivatized insulin in an aqueous solvent at acidic pH;
- b) adding a phenolic preservative;
- c) adding zinc;
- d) adding chloride anion to a final concentration of from about 15 mM to about 150 mM chloride anion above that introduced by pH adjustment;
- e) adding citrate to a concentration of from 1 mM to 10 mM;
- f) adding protamine;

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g) adjusting to a neutral pH;
and then holding the temperature of the neutral pH
suspension between about 20°C and about 37°C for between 12
hours and about 96 hours. For co-crystals, insulin is also
5 dissolved in the above process, and the proportion of
derivatized insulin relative to total protein is at least
50%. The crystals produced by this process are more
physically stable than crystals produced according to the
teachings of Brader I and Brader II, and they resuspend more
10 readily also. These are advantages to the patient.

The present invention is the discovery of the
dramatic and unexpected effect of chloride ions on particle
size of crystals and co-crystals, and on their physical
stability and re-suspendability. The ability to make
15 crystals and co-crystals having volume mean spherical
equivalent diameters in the range of about 1 micron to about
3 microns, which are ideally suited for pulmonary deposition
in high efficiency without additional particle
classification or size-reduction, came from the additional
20 discovery of the combined effects of chloride ion, higher
temperature, and low levels or absence of citrate.

The present invention solves two problems
currently not addressed by the art. First, previous
pulmonary methods for delivering insulin do not provide
25 adequate time action to control blood glucose between meals
and overnight. Second, presently known soluble, long-acting
insulins and insulin derivatives are delivered by
subcutaneous injection, which involves the inconvenience of
preparing a sample for injection, and the pain of a needle-
30 stick.

According to the present invention, a patient in
need of insulin to control blood glucose levels will benefit
from an advantageous slow uptake and prolonged persistence

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of insulin activity compared to inhalation of un-derivatized insulin, and also a reduction of inconvenience and pain compared with subcutaneous delivery. The crystals or co-crystals can be delivered in a carrier, as a solution or 5 suspension, or as a dry powder, using any of a variety of devices suitable for administration by inhalation. The acylated insulin can be administered using an inhalation device such as a nebulizer, a metered-dose inhaler, a dry powder inhaler, a sprayer, and the like.

10 The invention also provides a method for administering a pharmaceutical composition comprising either crystals or co-crystals and insulin or an insulin analog to a patient in need thereof by inhalation. Administering such combinations provides both post-prandial and basal control 15 of blood glucose levels. Because the method avoids injections, patient comfort is improved, and patient compliance increased compared with conventional insulin delivery methods.

20

Brief Description of the Drawing

Figure 1 depicts blood glucose levels following administration of crystals and co-crystals to F344 rats: intratracheal instillation of 1 mg/kg of 100% C8-BHI (solid squares, solid line); intratracheal instillation of 1 mg/kg 25 of 75% C8-BHI:25% BHI (open squares, solid line); subcutaneous administration of 1 mg/kg of 75% C8-BHI:25% BHI (solid circles, dashed line); and subcutaneous administration of 1 mg/kg of NPH insulin as control (solid triangles, solid line).

30

Detailed Description of the Invention

As used herein, the term "crystal" means a microcrystal comprising derivatized insulin or derivatized

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insulin analog, a divalent metal cation, a complexing compound, and a hexamer-stabilizing compound. Crystals of this type were originally described by Brader, M. [U.S. Patent Application No. 09/177685, filed 22 October 1998; 5 PCT/US98/22434; European Patent Publication No. 0911035, published 28 April 1999].

As used herein, the term "co-crystal" means a microcrystal comprising insulin or an insulin analog, derivatized insulin or derivatized insulin analog, a 10 divalent metal cation, a complexing compound, and a hexamer-stabilizing compound. Co-crystals of this type were originally described by Brader, M. [U.S. Patent Application No. 09/217275, filed 21 December 1998; PCT/US98/27299].

The term "microcrystal" means a solid that is 15 comprised primarily of matter in a crystalline state, and are of a microscopic size, typically of longest dimension within the range 1 micron to 100 microns. The term "microcrystalline" refers to the state of being a microcrystal.

20 The term "rod-like" means the distinctive microcrystal morphology that is also described as pyramidal-tipped tetragonal rods. The morphology of microcrystals of the present invention are easily determined by microscopic examination.

25 The term "protein" may have its common meaning, that is, a polymer of amino acids. The term "protein," as used herein, also has a narrower meaning; that is, a protein selected from the group consisting of insulin, insulin analogs, and proinsulins. The term "un-derivatized protein" 30 also refers to a protein selected from the group consisting of insulin, insulin analogs, and proinsulins.

As used in the claims, and elsewhere as the context dictates, the term "total protein" refers to the

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combined amount of protein (insulin, an insulin analog, or a proinsulin) and derivatized protein (derivatized insulin, a derivatized insulin analog, or a derivatized proinsulin). Although protamine and other known complexing compounds are 5 also proteins in the broadest sense of that term, the term "total protein" does not include them.

The term "derivatized protein" refers to a protein selected from the group consisting of derivatized insulin, and derivatized insulin analogs that is derivatized by a 10 functional group such that the derivatized protein is either less soluble in an aqueous solvent than is the un-derivatized protein, is more lipophilic than un-derivatized insulin, or produces a complex with zinc and protamine that is less soluble than the corresponding complex with the un-15 derivatized protein. The determination of either the solubility or lipophilicity of proteins and derivatized proteins is well-known to the skilled person. The solubility of derivatized proteins and protein in complexes with zinc and protamine can be readily determined by well-20 known procedures [Graham and Pomeroy, *J. Pharm. Pharmacol.* 36:427-430 (1983), as modified in DeFelippis, M. R. and Frank, B., EP 735,048], or the procedure used herein.

Many examples of such derivatized proteins are known in the art, including benzoyl, *p*-tolyl-sulfonamide 25 carbonyl, and indolyl derivatives of insulin and insulin analogs [Havelund, S., et al., WO95/07931, published 23 March 1995]; alkyloxycarbonyl derivatives of insulin [Geiger, R., et al., U.S. Patent No. 3,684,791, issued 15 August 1972; Brandenberg, D., et al., U.S. 3,907,763, issued 30 23 September 1975]; aryloxycarbonyl derivatives of insulin [Brandenberg, D., et al., U.S. 3,907,763, issued 23 September 1975]; alkylcarbamyl derivatives [Smyth, D. G., U.S. Patent No. 3,864,325, issued 4 February 1975; Lindsay,

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D. G., et al., U.S. Patent No. 3,950,517, issued 13 April 1976]; carbamyl, O-acetyl derivatives of insulin [Smyth, D. G., U.S. Patent No. 3,864,325 issued 4 February 1975]; cross-linked, alkyl dicarboxyl derivatives [Brandenberg, D., et al., U.S. Patent No. 3,907,763, issued 23 September 1975]; N-carbamyl, O-acetylated insulin derivatives [Smyth, D. G., U.S. Patent No. 3,868,356, issued 25 February 1975]; various O-alkyl esters [Markussen, J., U.S. Patent No. 4,343,898, issued 10 August 1982; Morihara, K., et al., U.S. Patent No. 4,400,465, issued 23 August 1983; Morihara, K., et al., U.S. Patent No. 4,401,757, issued 30 August 1983; Markussen, J., U.S. Patent No. 4,489,159, issued 18 December 1984; Obermeier, R., et al., U.S. Patent No. 4,601,852, issued 22 July 1986; and Andresen, F. H., et al., U.S. Patent No. 4,601,979, issued 22 July 1986]; alkylamide derivatives of insulin [Balschmidt, P., et al., U.S. Patent No. 5,430,016, issued 4 July 1995]; various other derivatives of insulin [Lindsay, D. G., U.S. Patent No. 3,869,437, issued 4 March 1975]; and the fatty acid-acylated proteins that are described herein.

The term "acylated protein" as used herein refers to a derivatized protein selected from the group consisting of insulin and insulin analogs that is acylated with an organic acid moiety that is bonded to the protein through an amide bond formed between the acid group of an organic acid compound and an amino group of the protein. In general, the amino group may be the α -amino group of an N-terminal amino acid of the protein, or may be the ϵ -amino group of a Lys residue of the protein. An acylated protein may be acylated at one or more of the three amino groups that are present in insulin and in most insulin analogs. Mono-acylated proteins are acylated at a single amino group. Di-acylated proteins are acylated at two amino groups. Tri-acylated proteins are

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acylated at three amino groups. The organic acid compound may be, for example, a fatty acid, an aromatic acid, or any other organic compound having a carboxylic acid group that will form an amide bond with an amino group of a protein, 5 and that will lower the aqueous solubility, raise the lipophilicity, or decrease the solubility of zinc/protamine complexes of the derivatized protein compared with the un-derivatized protein.

The term "fatty acid-acylated protein" refers to a 10 an acylated protein selected from the group consisting of insulin and insulin analogs that is acylated with a fatty acid that is bonded to the protein through an amide bond formed between the acid group of the fatty acid and an amino group of the protein. In general, the amino group may be 15 the α -amino group of an N-terminal amino acid of the protein, or may be the ϵ -amino group of a Lys residue of the protein. A fatty acid-acylated protein may be acylated at one or more of the three amino groups that are present in insulin and in most insulin analogs. Mono-acylated proteins 20 are acylated at a single amino group. Di-acylated proteins are acylated at two amino groups. Tri-acylated proteins are acylated at three amino groups. Fatty acid-acylated insulin is disclosed in a Japanese patent application 1-254,699.

See also, Hashimoto, M., et al., *Pharmaceutical Research*, 25 6:171-176 (1989), and Lindsay, D. G., et al., *Biochemical J.* 121:737-745 (1971). Further disclosure of fatty acid-acylated insulins and fatty acylated insulin analogs, and of methods for their synthesis, is found in Baker, J. C., et al, U.S. 08/342,931, filed 17 November 1994 and issued as 30 U.S. Patent No. 5,693,609, 2 December 1997; Havelund, S., et al., WO95/07931, published 23 March 1995, and a corresponding U.S. Patent No. 5,750,497, 12 May 1998; and

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Jonassen, I., et al., WO96/29342, published 26 September 1996.

The term "fatty acid-acylated protein" includes pharmaceutically acceptable salts and complexes of fatty acid-acylated proteins. The term "fatty acid-acylated protein" also includes preparations of acylated proteins wherein the population of acylated protein molecules is homogeneous with respect to the site or sites of acylation. For example, $\text{N}\varepsilon$ -mono-acylated protein, $\text{B}1\text{-N}\alpha$ -mono-acylated protein, $\text{A}1\text{-N}\alpha$ -mono-acylated protein, $\text{A}1, \text{B}1\text{-N}\alpha$ -di-acylated protein, $\text{N}\varepsilon, \text{A}1\text{-N}\alpha$ -di-acylated protein, and $\text{N}\varepsilon, \text{A}1, \text{B}1\text{-N}\alpha$ -tri-acylated protein are all encompassed within the term "fatty acid-acylated protein" for the purpose of the present invention. The term also refers to preparations wherein the population of acylated protein molecules has heterogeneous acylation. In the latter case, the term "fatty acid-acylated protein" includes mixtures of mono-acylated and di-acylated proteins, mixtures of mono-acylated and tri-acylated proteins, mixtures of di-acylated and tri-acylated proteins, and mixtures of mono-acylated, di-acylated, and tri-acylated proteins.

The term "insulin" as used herein, refers to human insulin, whose amino acid sequence and special structure are well-known. Human insulin is comprised of a twenty-one amino acid A-chain and a thirty-amino acid B-chain which are cross-linked by disulfide bonds. A properly cross-linked insulin contains three disulfide bridges: one between position 7 of the A-chain and position 7 of the B-chain, a second between position 20 of the A-chain and position 19 of the B-chain, and a third between positions 6 and 11 of the A-chain.

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The term "insulin analog" means proteins that have an A-chain and a B-chain that have substantially the same amino acid sequences as the A-chain and B-chain of human insulin, respectively, but differ from the A-chain and B-chain of human insulin by having one or more amino acid deletions, one or more amino acid replacements, and/or one or more amino acid additions that do not destroy the insulin activity of the insulin analog.

"Animal insulins" are analogs of human insulin, and therefore, are insulin analogs, as defined herein. Four such animal insulins are rabbit, pork, beef, and sheep insulin. The amino acid substitutions that distinguish these animal insulins from human insulin are presented below for the reader's convenience.

| | Amino Acid Position | | | |
|----------------|---------------------|-----------|------------|------------|
| | <u>A8</u> | <u>A9</u> | <u>A10</u> | <u>B30</u> |
| human insulin | Thr | Ser | Ile | Thr |
| rabbit insulin | Thr | Ser | Ile | Ser |
| pork insulin | Thr | Ser | Ile | Ala |
| beef insulin | Ala | Ser | Val | Ala |
| sheep insulin | Ala | Gly | Val | Ala |

15

Another type of insulin analog, "monomeric insulin analog" is well-known in the art. Monomeric insulin analogs are structurally very similar to human insulin, and have activity similar or equal to human insulin, but have one or more amino acid deletions, replacements or additions that tend to disrupt the contacts involved in dimerization and hexamerization which results in their having less tendency to associate to higher aggregation states. Monomeric insulin analogs are rapid-acting analogs of human insulin, and are disclosed, for example, in Chance, R. E., et al., U.S. patent No. 5,514,646, 7 May 1996; Brems, D. N., et al.

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Protein Engineering, 5:527-533 (1992); Brange, J. J. V., et al., EPO publication No. 214,826, published 18 March 1987; Brange, J. J. V., et al., U.S. Patent No. 5,618,913, 8 April 1997; and Brange, J., et al., *Current Opinion in Structural Biology* 1:934-940 (1991). An example of monomeric insulin analogs is described as human insulin wherein Pro at position B28 is substituted with Asp, Lys, Leu, Val, or Ala, and wherein Lys at position B29 is Lys or is substituted with Pro, and also, AlaB26-human insulin, des(B28-B30)-human insulin, and des(B27)-human insulin. The monomeric insulin analogs employed as derivatives in the present crystals, or employed un-derivatized in the solution phase of suspension formulations, are properly cross-linked at the same positions as is human insulin.

Another group of insulin analogs for use in the present invention are those wherein the isoelectric point of the insulin analog is between about 7.0 and about 8.0. These analogs are referred to as "pI-shifted insulin analogs." Examples of such insulin analogs include ArgB31,ArgB32-human insulin, GlyA21,ArgB31,ArgB32-human insulin, ArgA0,ArgB31,ArgB32-human insulin, and ArgA0,GlyA21,ArgB31,ArgB32-human insulin.

Another group of insulin analogs consists of insulin analogs that have one or more amino acid deletions that do not significantly disrupt the activity of the molecule. This group of insulin analogs is designated herein as "deletion analogs." For example, insulin analogs with deletion of one or more amino acids at positions B1-B3 are active. Likewise, insulin analogs with deletion of one or more amino acids at positions B28-B30 are active. Examples of "deletion analogs" include des(B30)-human insulin, desPhe(B1)-human insulin, des(B27)-human insulin, des(B28-B30)-human insulin, and des(B1-B3)-human insulin.

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The deletion analogs employed as derivatives in the present crystals, or employed un-derivatized in the solution phase of suspension formulations, are properly cross-linked at the same positions as is human insulin.

5 Amidated amino acids, and particularly asparagine residues in insulin, are known to be chemically unstable [Jorgensen, K. H., et al. U.S. Patent No. 5,008,241, issued 16 April, 1991; Dorschug, M., U.S. Patent No. 5,656,722, issued 12 August, 1997]. Particularly, they are prone to
10 deamidation and various rearrangement reactions under certain conditions that are well-known. Therefore, optionally, an insulin analog may be insulin or an insulin analog that has one or more of its amidated residues replaced with other amino acids for the sake of chemical
15 stability. For example, Asn or Gln may be replaced with a non-amidated amino acid. Preferred amino acid replacements for Asn or Gln are Gly, Ser, Thr, Asp or Glu. It is preferred to replace one or more Asn residues. In particular, AsnA18, AsnA21, or AsnB3, or any combination of
20 those residues may be replaced by Gly, Asp, or Glu, for example. Also, GlnA15 or GlnB4, or both, may be replaced by either Asp or Glu. Preferred replacements are Asp at B21, and Asp at B3. Also preferred are replacements that do not change the charge on the protein molecule, so that
25 replacement of Asn or Gln with neutral amino acids is also preferred.

30 The term "proinsulin" means a single-chain peptide molecule that is a precursor of insulin. Proinsulin may be converted to insulin or to an insulin analog by chemical or, preferably, enzyme-catalyzed reactions. In proinsulin, proper disulfide bonds are formed as described herein. Proinsulin comprises insulin or an insulin analog and a connecting bond or a connecting peptide. A connecting

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peptide has between 1 and about 35 amino acids. The connecting bond or connecting peptide connects to a terminal amino acid of the A-chain and to a terminal amino acid of the B-chain by an α -amide bond or by two α -amide bonds, 5 respectively. Preferably, none of the amino acids in the connecting peptide is cysteine. Preferably, the C-terminal amino acid of the connecting peptide is Lys or Arg. Proinsulin may have the formula X-B-C-A-Y or may have the formula X-A-C-B-Y, wherein X is hydrogen or is a peptide of 10 from 1 to about 100 amino acids that has either Lys or Arg at its C-terminal amino acid, Y is hydroxy, or is a peptide of from 1 to about 100 amino acids that has either Lys or Arg at its N-terminal amino acid, A is the A-chain of insulin or the A-chain of an insulin analog, C is a peptide 15 of from 1 to about 35 amino acids, none of which is cysteine, wherein the C-terminal amino acid is Lys or Arg, and B is the B-chain of insulin or the B-chain of an insulin analog.

A "pharmaceutically acceptable salt" means a salt 20 formed between any one or more of the charged groups in a protein and any one or more pharmaceutically acceptable, non-toxic cations or anions. Organic and inorganic salts include, for example, those prepared from acids such as hydrochloric, sulfuric, sulfonic, tartaric, fumaric, 25 hydrobromic, glycolic, citric, maleic, phosphoric, succinic, acetic, nitric, benzoic, ascorbic, p-toluenesulfonic, benzenesulfonic, naphthalenesulfonic, propionic, carbonic, and the like, or for example, ammonium, sodium, potassium, calcium, or magnesium.

30 The verb "acylate" means to form the amide bond between a fatty acid and an amino group of a protein. A protein is "acylated" when one or more of its amino groups

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is combined in an amide bond with the acid group of a fatty acid.

The term "fatty acid" means a saturated or unsaturated, straight chain or branched chain fatty acid, 5 having from one to eighteen carbon atoms.

The term "C1 to C18 fatty acid" refers to a saturated, straight chain or branched chain fatty acid having from one to eighteen carbon atoms.

The term "divalent metal cation" refers to the ion 10 or ions that participate to form a complex with a multiplicity of protein molecules. The transition metals, the alkaline metals, and the alkaline earth metals are examples of metals that are known to form complexes with insulin. The transitional metals are preferred. Zinc is 15 particularly preferred. Other transition metals that may be pharmaceutically acceptable for complexing with insulin proteins include copper, cobalt, and iron.

The term "complex" has two meanings in the present invention. In the first, the term refers to a complex 20 formed between one or more atoms in the proteins that form the complex and one or more divalent metal cations. The atoms in the proteins serve as electron-donating ligands. The proteins typically form a hexamer complex with divalent transition metal cations. The second meaning of "complex" 25 in the present invention is the association between the complexing compound and hexamers. The "complexing compound" is an organic molecule that typically has a multiplicity of positive charges that binds to, or complexes with hexamers in the insoluble composition, thereby stabilizing them 30 against dissolution. Examples of complexing compounds suitable in the present invention include protamine, surfen, various globin proteins [Brange, J. , Galenics of Insulin, Springer-Verlag, Berlin Heidelberg (1987)], and various

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polycationic polymer compounds known to complex with insulin.

The term "protamine" refers to a mixture of strongly basic proteins obtained from fish sperm. The 5 average molecular weight of the proteins in protamine is about 4,200 [Hoffmann, J. A., et al., *Protein Expression and Purification*, 1:127-133 (1990)]. "Protamine" can refer to a relatively salt-free preparation of the proteins, often called "protamine base." Protamine also refers to 10 preparations comprised of salts of the proteins. Commercial preparations vary widely in their salt content.

Protamines are well-known to those skilled in the insulin art and are currently incorporated into NPH insulin products. A pure fraction of protamine is operable in the 15 present invention, as well as mixtures of protamines. Commercial preparations of protamine, however, are typically not homogeneous with respect to the proteins present. These are nevertheless operative in the present invention.

Protamine comprised of protamine base is operative in the 20 present invention, as are protamine preparations comprised of salts of protamine, and those that are mixtures of protamine base and protamine salts. Protamine sulfate is a frequently used protamine salt. All mass ratios referring to protamine are given with respect to protamine free base. 25 The person of ordinary skill can determine the amount of other protamine preparations that would meet a particular mass ratio referring to protamine.

The term "suspension" refers to a mixture of a liquid phase and a solid phase that consists of insoluble or 30 sparingly soluble particles that are larger than colloidal size. Mixtures of NPH microcrystals and an aqueous solvent form suspensions. The term "suspension formulation" means a pharmaceutical composition wherein an active agent is

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present in a solid phase, for example, a microcrystalline solid, an amorphous precipitate, or both, which is finely dispersed in an aqueous solvent. The finely dispersed solid is such that it may be suspended in a fairly uniform manner 5 throughout the aqueous solvent by the action of gently agitating the mixture, thus providing a reasonably uniform suspension from which a dosage volume may be extracted. Examples of commercially available insulin suspension 10 formulations include, for example, NPH, PZI, and Ultralente. 10 A small proportion of the solid matter in a microcrystalline suspension formulation may be amorphous. Preferably, the proportion of amorphous material is less than 10%, and most 15 preferably, less than 1% of the solid matter in a microcrystalline suspension. Likewise, a small proportion 15 of the solid matter in an amorphous precipitate suspension may be microcrystalline.

"NPH insulin" refers to the "Neutral Protamine Hagedorn" preparation of insulin. Synonyms include human insulin NPH and insulin NPH, among many others. Humulin® N 20 is a commercial preparation of NPH insulin. A related term is "NPL" which refers to an NPH-like preparation of LysB28, ProB29-human insulin analog. The meaning of these 25 terms, and the methods for preparing them will be familiar to the person of ordinary skill in the insulin formulation art.

The term "aqueous solvent" refers to a liquid solvent that contains water. An aqueous solvent system may be comprised solely of water, may be comprised of water plus one or more miscible solvents, and may contain solutes. The 30 more commonly-used miscible solvents are the short-chain organic alcohols, such as, methanol, ethanol, propanol, short-chain ketones, such as acetone, and polyalcohols, such as glycerol.

An "isotonicity agent" is a compound that is physiologically tolerated and imparts a suitable tonicity to a formulation to prevent the net flow of water across cell membranes that are in contact with an administered 5 formulation. Glycerol, which is also known as glycerin, is commonly used as an isotonicity agent. Other isotonicity agents include salts, e.g., sodium chloride, and monosaccharides, e.g., dextrose and lactose.

The crystals and co-crystals of the present 10 invention contain a hexamer-stabilizing compound. The term "hexamer-stabilizing compound" refers to a non-proteinaceous, small molecular weight compound that stabilizes the protein or derivatized protein in a hexameric association state. Phenolic compounds, particularly 15 phenolic preservatives, are the best known stabilizing compounds for insulin and insulin derivatives. Hexamer-stabilizing compounds stabilize the hexamer by binding to it through specific inter-molecular contacts. Examples of hexamer-stabilizing agents include: various phenolic 20 compounds, phenolic preservatives, resorcinol, 4'-hydroxyacetanilide, 4-hydroxybenzamide, and 2,7-dihydroxynaphthalene. Multi-use formulations of the insoluble compositions of the present invention will contain a preservative, in addition to a hexamer-stabilizing 25 compound. The preservative used in formulations of the present invention may be a phenolic preservative, and may be the same as, or different from the hexamer-stabilizing compound.

The term "preservative" refers to a compound added 30 to a pharmaceutical formulation to act as an anti-microbial agent. A parenteral formulation must meet guidelines for preservative effectiveness to be a commercially viable multi-use product. Among preservatives known in the art as

being effective and acceptable in parenteral formulations are benzalkonium chloride, benzethonium, chlorohexidine, phenol, m-cresol, benzyl alcohol, methylparaben, chlorobutanol, o-cresol, p-cresol, chlorocresol, 5 phenylmercuric nitrate, thimerosal, benzoic acid, and various mixtures thereof. See, e.g., Wallhäuser, K.-H., *Develop. Biol. Standard*, 24:9-28 (1974) (S. Kräger, Basel).

The term "phenolic preservative" includes the compounds phenol, m-cresol, o-cresol, p-cresol, 10 chlorocresol, methylparaben, and mixtures thereof. Certain phenolic preservatives, such as phenol and m-cresol, are known to bind to insulin-like molecules and thereby to induce conformational changes that increase either physical or chemical stability, or both [Birnbaum, D. T., et al., 15 *Pharmaceutical. Res.* 14:25-36 (1997); Rahuel-Clermont, S., et al., *Biochemistry* 36:5837-5845 (1997)].

The term "buffer" or "pharmaceutically acceptable buffer" refers to a compound that is known to be safe for use in insulin formulations and that has the effect of 20 controlling the pH of the formulation at the pH desired for the formulation. The pH of the formulations of the present invention is from about 6.0 to about 8.0. Preferably the formulations of the present invention have a pH between about 6.8 and about 7.8. Pharmaceutically acceptable 25 buffers for controlling pH at a moderately acidic pH to a moderately basic pH include such compounds as phosphate, acetate, citrate, arginine, TRIS, and histidine. "TRIS" refers to 2-amino-2-hydroxymethyl-1,3,-propanediol, and to any pharmacologically acceptable salt thereof. The free 30 base and the hydrochloride form are two common forms of TRIS. TRIS is also known in the art as trimethylol aminomethane, tromethamine, and tris(hydroxymethyl)aminomethane. Other buffers that are

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pharmaceutically acceptable, and that are suitable for controlling pH at the desired level are known to the chemist of ordinary skill.

The term "administer" means to introduce a 5 formulation of the present invention into the body of a patient in need thereof to treat a disease or condition.

The term "treating" refers to the management and care of a patient having diabetes or hyperglycemia, or other condition for which insulin administration is indicated for 10 the purpose of combating or alleviating symptoms and complications of those conditions. Treating includes administering a formulation of present invention to prevent the onset of the symptoms or complications, alleviating the symptoms or complications, or eliminating the disease, 15 condition, or disorder.

The abbreviations "MMAD" and "MMEA" are well-known in the art, and stand for "mass median aerodynamic diameter" and "mass median equivalent aerodynamic diameter," respectively. The terms are substantially equivalent. The 20 "aerodynamic equivalent" size of a particle is the diameter of a unit density sphere which exhibits the same aerodynamic behavior as the particle, regardless of actual density or shape. MMAD is determined using a cascade impactor, which measures the particle size as a function of the aerodynamic behavior of the particle in a high velocity airstream. The 25 median (50%) particle size is obtained from a linear regression analysis of the cumulative distribution data.

The abbreviation "VMED" stands for "volume mean spherical equivalent diameter." This term is well-known in 30 the art of particle sizing.

The crystals and co-crystals of the present invention have rod-like morphology or an irregular morphology. Preferably, the crystals or co-crystals are

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comprised of acylated insulin or acylated insulin analog, zinc ions, which are present at about 0.3 to about 0.7 mole per mole of total protein, a phenolic preservative selected from the group consisting of phenol, m-cresol, o-cresol, p-cresol, chlorocresol, and mixtures thereof and is present in sufficient proportions with respect to total protein to stabilize the T3R3 or R6 hexamer conformation, and protamine, which is present at about 0.15 to about 0.7 mg per 3.5 mg of total protein.

10 A preferred group of insulin analogs for preparing derivatized insulin analogs used to form crystals and co-crystals consists of animal insulins, deletion analogs, and pI-shifted analogs. A more preferred group consists of animal insulins and deletion analogs. Deletion analogs are 15 yet more preferred.

Another preferred group of insulin analogs for use in the crystals and co-crystals of the present invention consists of the monomeric insulin analogs. Particularly preferred are those monomeric insulin analogs wherein the 20 amino acid residue at position B28 is Asp, Lys, Leu, Val, or Ala, the amino acid residue at position B29 is Lys or Pro, the amino acid residue at position B10 is His or Asp, the amino acid residue at position B1 is Phe, Asp or deleted alone or in combination with a deletion of the residue at 25 position B2, the amino acid residue at position B30 is Thr, Ala, Ser, or deleted, and the amino acid residue at position B9 is Ser or Asp; provided that either position B28 or B29 is Lys.

Another preferred group of insulin analogs for use 30 in the present invention consists of those wherein the isoelectric point of the insulin analog is between about 7.0 and about 8.0. These analogs are referred to as "pI-shifted insulin analogs." Examples of pI-shifted insulin analogs

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include, for example, ArgB31,ArgB32-human insulin, GlyA21,ArgB31,ArgB32-human insulin, ArgA0,ArgB31,ArgB32-human insulin, and ArgA0,GlyA21,ArgB31,ArgB32-human insulin.

Another preferred group of insulin analogs 5 consists of LysB28,ProB29-human insulin (B28 is Lys; B29 is Pro); AspB28-human insulin (B28 is Asp), AspB1-human insulin, ArgB31,ArgB32-human insulin, ArgA0-human insulin, AspB1,GluB13-human insulin, AlaB26-human insulin, GlyA21-human insulin, des(ThrB30)-human insulin, and 10 GlyA21,ArgB31,ArgB32-human insulin.

Especially preferred insulin analogs include LysB28,ProB29-human insulin, des(ThrB30)-human insulin, AspB28-human insulin, and AlaB26-human insulin. Another especially preferred insulin analog is GlyA21, ArgB31, 15 ArgB32-human insulin [Dörschug, M., U. S. Patent No. 5,656,722, 12 August 1997]. The most preferred insulin analog is LysB28,ProB29-human insulin.

The preferred derivatized proteins are acylated proteins, and the preferred acylated proteins for the 20 microcrystals and formulations of the present invention are fatty acid-acylated insulin and fatty acid-acylated insulin analogs. Fatty acid-acylated human insulin is highly preferred. Fatty acid-acylated insulin analogs are also highly preferred.

25 The particular group used to derivatize insulin or an insulin analog (collectively, protein) may be any chemical moiety that does not significantly reduce the biological activity of the protein, is not toxic when bonded to the protein, and most importantly, reduces the aqueous 30 solubility, raises the lipophilicity, or decreases the solubility of zinc/protamine complexes of the derivatized protein.

One preferred group of acylating moieties consists of fatty acids that are straight chain and saturated. This group consists of methanoic acid (C1), ethanoic acid (C2), propanoic acid (C3), n-butanoic acid (C4), n-pentanoic acid 5 (C5), n-hexanoic acid (C6), n-heptanoic acid (C7), n-octanoic acid (C8), n-nonanoic acid (C9), n-decanoic acid (C10), n-undecanoic acid (C11), n-dodecanoic acid (C12), n-tridecanoic acid (C13), n-tetradecanoic acid (C14), n-pentadecanoic acid (C15), n-hexadecanoic acid (C16), n-heptadecanoic acid (C17), and n-octadecanoic acid (C18). Adjectival forms are formyl (C1), acetyl (C2), propionyl (C3), butyryl (C4), pentanoyl (C5), hexanoyl (C6), heptanoyl (C7), octanoyl (C8), nonanoyl (C9), decanoyl (C10), undecanoyl (C11), dodecanoyl (C12), tridecanoyl (C13), 15 tetradecanoyl (C14) or myristoyl, pentadecanoyl (C15), hexadecanoyl (C16) or palmitic, heptadecanoyl (C17), and octadecanoyl (C18) or stearic.

A preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of 20 the present invention consists of fatty acids having an even number of carbon atoms - that is, C2, C4, C6, C8, C10, C12, C14, C16, and C18 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 25 of the present invention consists of fatty acids having an odd number of carbon atoms - that is, C1, C3, C5, C7, C9, C11, C13, C15, and C17 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 30 of the present invention consists of fatty acids having more than 5 carbon atoms - that is, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, C16, C17, and C18 saturated fatty acids.

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Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having less than 9 carbon atoms - that is, C1, C2, C3, C4, C5, C6, C7, 5 and C8 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having between 6 and 8 carbon atoms - that is, C6, C7, and C8, 10 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having more than between 4 and 6 carbon atoms - that is, C4, C5, and C6, 15 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having more than between 2 and 4 carbon atoms - that is, C2, C3, and C4, 20 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having less than 6 carbon atoms - that is, C1, C2, C3, C4, and C5 25 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having less than 4 carbon atoms - that is, C1, C2, and C3 saturated 30 fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having more

than 9 carbon atoms - that is, C10, C11, C12, C13, C14, C15, C16, C17, and C18 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 5 of the present invention consists of fatty acids having an even number of carbon atoms and more than 9 carbon atoms - that is, C10, C12, C14, C16, and C18 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 10 of the present invention consists of fatty acids having 12, 14, or 16 carbon atoms, that is, C12, C14, and C16 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 15 of the present invention consists of fatty acids having 14 or 16 carbon atoms, that is, C14 and C16 saturated fatty acids. Fatty acids with 14 carbons are particularly preferred. Fatty acids with 16 carbons are also particularly preferred.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 20 of the present invention consists of saturated fatty acids having between 4 and 10 carbon atoms, that is C4, C5, C6, C7, C8, C9, and C10 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 25 of the present invention consists of saturated fatty acids having an even number of carbon atoms between 4 and 10 carbon atoms, that is C4, C6, C8, and C10 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals 30 of the present invention consists of fatty acids having 6,

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8, or 10 carbon atoms. Fatty acids with 6 carbons are particularly preferred. Fatty acids with 8 carbons are also particularly preferred. Fatty acids with 10 carbons are particularly preferred.

5 The skilled person will appreciate that narrower preferred groups are made by combining the preferred groups of fatty acids described above.

Another preferred group of acylating moieties consists of saturated fatty acids that are branched. A 10 branched fatty acid has at least two branches. The length of a "branch" of a branched fatty acid may be described by the number of carbon atoms in the branch, beginning with the acid carbon. For example, the branched fatty acid 3-ethyl-5-methylhexanoic acid has three branches that are five, six, 15 and six carbons in length. In this case, the "longest" branch is six carbons. As another example, 2,3,4,5-tetraethyloctanoic acid has five branches that are 4, 5, 6, 7, and 8 carbons long. The "longest" branch is eight carbons. A preferred group of branched fatty acids are 20 those having from three to ten carbon atoms in the longest branch.

A representative number of such branched, saturated fatty acids will be mentioned to assure the reader's comprehension of the range of such fatty acids that 25 may be used as acylating moieties of the proteins in the present invention: 2-methyl-propioinic acid, 2-methyl-butyric acid, 3-methyl-butyric acid, 2,2-dimethyl-propionic acid, 2-methyl-pentanoic acid, 3-methyl-pentanoic acid, 4-methyl-pentanoic acid, 2,2-dimethyl-butyric acid, 2,3-dimethyl-butyric acid, 3,3-dimethyl-butyric acid, 2-ethyl-butyric acid, 2-methyl-hexanoic acid, 5-methyl-hexanoic acid, 2,2-dimethyl-pentanoic acid, 2,4-dimethyl-pentanoic acid, 2-ethyl-3-methyl-butyric acid, 2-ethyl-pentanoic acid,

3-ethyl-pentanoic acid, 2,2-dimethyl-3-methyl-butyric acid, 2-methyl-heptanoic acid, 3-methyl-heptanoic acid, 4-methyl-heptanoic acid, 5-methyl-heptanoic acid, 6-methyl-heptanoic acid, 2,2-dimethyl-hexanoic acid, 2,3-dimethyl-hexanoic acid, 2,4-dimethyl-hexanoic acid, 2,5-dimethyl-hexanoic acid, 3,3,-dimethyl-hexanoic acid, 3,4-dimethyl-hexanoic acid, 3,5-dimethyl-hexanoic acid, 4,4-dimethyl-hexanoic acid, 2-ethyl-hexanoic acid, 3-ethyl-hexanoic acid, 4-ethyl-hexanoic acid, 2-propyl-pentanoic acid, 2-ethyl-hexanoic acid, 3-ethyl-hexanoic acid, 4-ethyl-hexanoic acid, 2-(1-propyl)pentanoic acid, 2-(2-propyl)pentanoic acid, 2,2-diethyl-butyric acid, 2,3,4-trimethyl-pentanoic acid, 2-methyl-octanoic acid, 4-methyl-octanoic acid, 7-methyl-octanoic acid, 2,2-dimethyl-heptanoic acid, 2,6-dimethyl-heptanoic acid, 2-ethyl-2-methyl-hexanoic acid, 3-ethyl-5-methyl-hexanoic acid, 3-(1-propyl)-hexanoic acid, 2-(2-butyl)-pentanoic acid, 2-(2-(2-methylpropyl))pentanoic acid, 2-methyl-nonanoic acid, 8-methyl-nonanoic acid, 6-ethyl-octanoic acid, 4-(1-propyl)-heptanoic acid, 5-(2-propyl)-heptanoic acid, 3-methyl-undecanoic acid, 2-pentyl-heptanoic acid, 2,3,4,5,6-pentamethyl-heptanoic acid, 2,6-diethyl-octanoic acid, 2-hexyl-octanoic acid, 2,3,4,5,6,7-hexamethyl-octanoic acid, 3,3-diethyl-4,4-diethyl-hexanoic acid, 2-heptyl-nonanoic acid, 2,3,4,5-tetraethyl-octanoic acid, 2-octyl-decanoic acid, and 2-(1-propyl)-3-(1-propyl)-4,5-diethyl-6-methyl-heptanoic acid.

Yet another preferred group of acylating moieties consists of cyclic alkyl acids having from 5 to 24 carbon atoms, wherein the cyclic alkyl moiety, or moieties, have 5 to 7 carbon atoms. A representative number of such cyclic alkyl acids will be mentioned to assure the reader's comprehension of the range of such acids that may be used as acylating moieties of the proteins in the present invention:

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cyclopentyl-formic acid, cyclohexyl-formic acid, 1-cyclopentyl-acetic acid, 2-cyclohexyl-acetic acid, 1,2-dicyclopentyl-acetic acid, and the like.

A preferred group of derivatized proteins consists 5 of mono-acylated proteins. Mono-acylation at the ϵ -amino group is most preferred. For insulin, mono-acylation at LysB29 is preferred. Similarly, for certain insulin analogs, such as, LysB28, ProB29-human insulin analog, mono-acylation at the ϵ -amino group of LysB28 is most preferred. 10 Mono-acylation at the α -amino group of the B-chain (B1) is also preferred. Mono-acylation at the α -amino group of the A-chain (A1) is also preferred.

Another group of acylated proteins consists of di-acylated proteins. The di-acylation may be, for example, at 15 the ϵ -amino group of Lys and at the α -amino group of the B-chain, or may be at the ϵ -amino group of Lys and at the α -amino group of the A-chain, or may be at the α -amino group of the A-chain and at the α -amino group of the B-chain.

Another group of acylated proteins consists of 20 tri-acylated proteins. Tri-acylated proteins are those that are acylated at the ϵ -amino group of Lys, at the α -amino group of the B-chain, and at the α -amino group of the A-chain.

Aqueous compositions containing water as the major 25 solvent are preferred. Aqueous suspensions wherein water is the solvent are highly preferred.

The compositions of the present invention are used to treat patients who have diabetes or hyperglycemia. The formulations of the present invention will typically provide 30 derivatized protein at concentrations of from about 1 mg/mL to about 10 mg/mL. Present formulations of insulin products

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are typically characterized in terms of the concentration of units of insulin activity (units/mL), such as U40, U50, U100, and so on, which correspond roughly to about 1.4, 1.75, and 3.5 mg/mL preparations, respectively. The dose, 5 route of administration, and the number of administrations per day will be determined by a physician considering such factors as the therapeutic objectives, the nature and cause of the patient's disease, the patient's gender and weight, level of exercise, eating habits, the method of 10 administration, and other factors known to the skilled physician. In broad range, a daily dose would be in the range of from about 1 nmol/kg body weight to about 6 nmol/kg body weight (6 nmol is considered equivalent to about 1 unit of insulin activity). A dose of between about 2 and about 3 15 nmol/kg is typical of present insulin therapy.

The physician of ordinary skill in treating diabetes will be able to select the therapeutically most advantageous means to administer the formulations of the present invention. Parenteral routes of administration are 20 preferred. Typical routes of parenteral administration of suspension formulations of insulin are the subcutaneous and intramuscular routes. The compositions and formulations of the present invention may also be administered by nasal, buccal, pulmonary, or ocular routes. The pulmonary route 25 is particularly advantageous, in that pain and inconvenience are reduced. The crystals and co-crystals of the present invention are particularly well-suited for pulmonary administration.

Glycerol at a concentration of 12 mg/mL to 25 30 mg/mL is preferred as an isotonicity agent. Yet more highly preferred for isotonicity is to use glycerol at a concentration of from about 15 mg/mL to about 17 mg/mL.

M-cresol and phenol, or mixtures thereof, are preferred preservatives in formulations of the present invention.

Insulin or insulin analogs used to prepare derivatized proteins can be prepared by any of a variety of recognized peptide synthesis techniques including classical (solution) methods, solid phase methods, semi-synthetic methods, and more recent recombinant DNA methods. For example, see Chance, R. E., et al., U.S. Patent No. 5,514,646, 7 May 1996; EPO publication number 383,472, 7 February 1996; Brange, J. J. V., et al. EPO publication number 214,826, 18 March 1987; and Belagaje, R. M., et al., U.S. Patent No. 5,304,473, 19 April 1994, which disclose the preparation of various proinsulin and insulin analogs. These references are expressly incorporated herein by reference.

Generally, derivatized proteins are prepared using methods known in the art. The publications listed above to describe derivatized proteins contain suitable methods to prepare derivatized proteins. Those publications are expressly incorporated by reference for methods of preparing derivatized proteins. To prepare acylated proteins, the protein is reacted with an activated organic acid, such as an activated fatty acid. Activated fatty acids are derivatives of commonly employed acylating agents, and include activated esters of fatty acids, fatty acid halides, activated amides of fatty acids, such as, activated azolide derivatives [Hansen, L. B., WIPO Publication No. 98/02460, 22 January 1998], and fatty acid anhydrides. The use of activated esters, especially N-hydroxysuccinimide esters of fatty acids, is a particularly advantageous means of acylating a free amino acid with a fatty acid. Lapidot, et al. describe the preparation of N-hydroxysuccinimide esters

and their use in the preparation of N-lauroyl-glycine, N-lauroyl-L-serine, and N-lauroyl-L-glutamic acid. The term "activated fatty acid ester" means a fatty acid which has been activated using general techniques known in the art

5 [Riordan, J. F. and Vallee, B. L., *Methods in Enzymology*, XXV:494-499 (1972); Lapidot, Y., et al., *J. Lipid Res.* 8:142-145 (1967)]. Hydroxybenzotriazole (HOBT), N-hydroxysuccinimide and derivatives thereof are particularly well known for forming activated acids for peptide

10 synthesis.

To selectively acylate the ϵ -amino group, various protecting groups may be used to block the α -amino groups during the coupling. The selection of a suitable protecting group is known to one skilled in the art and includes *p*-methoxybenzoxycarbonyl (pmZ). Preferably, the ϵ -amino group is acylated in a one-step synthesis without the use of amino-protecting groups. A process for selective acylation at the $\text{N}\epsilon$ -amino group of Lys is disclosed and claimed by Baker, J. C., et al., U.S. Patent No. 5,646,242, 8 July 1997, the entire disclosure of which is incorporated expressly by reference. A process for preparing a dry powder of an acylated protein is disclosed and claimed by Baker, J. C., et al., U.S. Patent No. 5,700,904, 23 December 1997, the entire disclosure of which is incorporated herein expressly by reference.

The primary role of zinc in the present invention is to facilitate formation of Zn(II) hexamers of the protein and derivatized protein, either separately as mixed hexamers, or together as hybrid hexamers. Zinc facilitates

30 the formation of hexamers of insulin, and of insulin analogs. Zinc likewise promotes the formation of hexamers of derivatized insulin and insulin analogs. Hexamer

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formation is conveniently achieved by bringing the pH of a solution comprising protein, or derivatized protein, or both into the neutral region in the presence of Zn(II) ions, or by adding Zn(II) after the pH has been adjusted to the 5 neutral region.

For efficient yield of crystals or co-crystals, the molar ratio of zinc to total protein is bounded at the lower limit by about 0.33, that is, the approximately two zinc atoms per hexamer which are needed for efficient 10 hexamerization. Crystals and co-crystals will form suitably with about 2 to about 4-6 zinc atoms present when no compound that competes with insulin for zinc binding is present. Even more zinc may be used during the process if a compound that competes with the protein for zinc binding, 15 such as one containing citrate or phosphate, is present. Excess zinc above the minimum amount needed for efficient hexamerization may be desirable to more strongly drive hexamerization. Also, excess zinc above the minimum amount can be present in a formulation of the present invention, 20 and may be desirable to improve chemical and physical stability, to improve suspendability, and possibly to further extend time-action. Consequently, there is a fairly wide range of zinc:protein ratios allowable in the insoluble compositions, processes, and formulations of the present 25 invention.

In accordance with the present invention, zinc is present in the formulation in an amount of from about 0.3 mole to about 7 moles per mole of total protein and more preferably about from 0.3 mole to about 1.0 mole of total 30 protein. Yet more highly preferred is a ratio of zinc to derivatized protein from about 0.3 to about 0.7 mole of zinc atoms per mole of total protein. Most highly preferred is a ratio of zinc to total protein from about 0.30 to about 0.55

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mole of zinc atoms per mole of total protein. For higher zinc formulations that are similar to PZI preparations, the zinc ratio is from about 5 to about 7 moles of zinc per mole of total protein.

5 The zinc compound that provides zinc for the present invention may be any pharmaceutically acceptable zinc compound. The addition of zinc to insulin preparations is known in the art, as are pharmaceutically acceptable sources of zinc. Preferred zinc compounds to supply zinc
10 for the present invention include zinc chloride, zinc acetate, zinc citrate, zinc oxide, and zinc nitrate.

15 A complexing compound is required for the microcrystals and precipitates of the present invention. The complexing compound must be present in sufficient quantities to cause substantial precipitation and crystallization of the hexamers. Such quantities can be readily determined for a particular preparation of a particular complexing compound by simple titration experiments. Ideally, the complexing compound concentration
20 is adjusted so that there is negligible complexing compound remaining in the soluble phase after completion of precipitation and crystallization. This requires combining the complexing compound based on an experimentally determined "isophane" ratio. This ratio is expected to be
25 very similar to that of NPH and NPL. However, it may be slightly different because derivatization may affect the nature of the protein-protamine interaction.

30 When protamine is the complexing compound, it is present in the crystals and co-crystals in an amount of from about 0.15 mg to about 0.5 mg per 3.5 mg of the total protein. The ratio of protamine to total protein is preferably from about 0.25 to about 0.40 (mg/3.5 mg). More preferably the ratio is from about 0.25 to about 0.38

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(mg/3.5 mg). Preferably, protamine is in an amount of 0.05 mg to about 0.2 mg per mg of the total protein, and more preferably, from about 0.05 to about 0.15 milligram of protamine per milligram of total protein. Protamine sulfate 5 is the preferred salt form of protamine for use in the present invention. When protamine sulfate, or other salt form of protamine is used, the mass of it to be used would have to be adjusted with respect to the mass of protamine free base that would be used for the same application by a 10 factor equal to the ratio of the molecular weights of the salt form and protamine.

To further extend the time action of the compositions of the present invention or to improve their suspendability, additional protamine and zinc may be added 15 after crystallization. Thus, also within the present invention are formulations having protamine at higher than isophane ratios. For these formulations, the protamine ratio is from 0.25 mg to about 0.5 mg of protamine per mg of total protein.

20 A required component of the crystals and co-crystals of the present invention is a hexamer stabilizing compound. The structures of three hexameric conformations have been characterized in the literature, and are designated T6, T3R3, and R6. In the presence of hexamer 25 stabilizing compound, such as various phenolic compounds, the R6 conformation is stabilized. Therefore, it is highly likely that hexamers are in the R6 conformation, or the T3R3 conformation in the crystals and co-crystals produced in the presence of a hexamer stabilizing compound, such as phenol 30 or m-cresol, among others. A wide range of hexamer stabilizing compounds are suitable. They must be present in sufficient proportions with respect to total protein to stabilize the R6 hexamer conformation. To accomplish this,

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at least 3 moles of hexamer stabilizing compound per hexamer are required for effective hexamer stabilization. It is preferred that more than 3 moles of hexamer stabilizing compound per hexamer be present in the crystals and co-crystals of the present invention. The presence of higher ratios of hexamer stabilizing compound, at least up to 25 to 50-fold higher, in the solution from which the microcrystals and precipitates are prepared will not adversely affect hexamer stabilization.

In formulations of the present invention, a preservative may be present, especially if the formulation is intended to be sampled multiple times. As mentioned above, a wide range of suitable preservatives are known. Preferably, the preservative is present in the solution in an amount suitable to provide an antimicrobial effect sufficient to meet pharmacopoeial requirements.

Preferred preservatives are the phenolic preservatives, which are enumerated above. Preferred concentrations for the phenolic preservative are from about 2 mg to about 5 mg per milliliter of the aqueous suspension formulation. These concentrations refer to the total mass of phenolic preservatives because mixtures of individual phenolic preservatives are contemplated. Suitable phenolic preservatives include, for example, phenol, m-cresol, and methylparaben. Preferred phenolic compounds are phenol and m-cresol. Mixtures of phenolic compounds, such as phenol and m-cresol, are also contemplated and highly preferred. Examples of mixtures of phenolic compounds are 0.6 mg/mL phenol and 1.6 mg/mL m-cresol, and 0.7 mg/mL phenol and 1.8 mg/mL m-cresol.

The crystals and co-crystals of the present invention are preferably oblong-shaped, also known as "rod-like", single crystals that are comprised of a derivatized

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protein, a divalent metal cation, and including a complexing compound and a hexamer-stabilizing compound.

A preferred composition comprises from about 3 mg to about 6 mg of protamine sulfate per 35 mg of total protein, and from about 0.1 to about 0.4 mg zinc per 35 mg of total protein. Another preferred composition comprises from about 10 mg to about 17 mg of protamine sulfate per 35 mg of total protein, and from about 2.0 to about 2.5 mg zinc per 35 mg of total protein. Another preferred composition comprises, per mL, protamine sulfate, 0.34-0.38 mg; zinc, 0.01-0.04 mg; and total protein, 3.2-3.8 mg.

Both an un-derivatized protein and a derivatized protein are required for the present co-crystals. The ratio between the masses of these proteins determines the degree of time extension of the preparations. A preferred ratio of the number of moles of the protein to the number of moles of the derivatized protein is between about 1:100 and about 100:1. A further preferred ratio of the number of moles of the protein to the number of moles of the derivatized protein is between about 1:1 and about 100:1. Another preferred ratio of the number of moles of the protein to the number of moles of the derivatized protein is between about 1:1 and about 20:1. Yet other preferred ratios of the number of moles of the protein to the number of moles of the derivatized protein are: between about 2:1 and about 20:1; between about 2:1 and 10:1; between about 2:1 and 5:1; between about 3:1 and 5:1; between 1:1 and 1:20; between 1:1 and 1:10; between about 1:2 and about 1:20; between about 1:2 and 1:10; between about 1:2 and 1:5; between about 1:3 and 1:5; between about 10:1 and about 1:10; between about 9:1 and about 1:9; between about 5:1 and about 1:5; and between about 3:1 and about 1:3.

The present invention provides processes for preparing the crystals and co-crystals. In summary, suitable processes are comprised generally of the steps in one of the following sequences: solubilization (if starting 5 with dry material), hexamerization, homogenization, complexation, precipitation, crystallization, and optionally formulation; or solubilization (if starting with dry material), homogenization, hexamerization, complexation, precipitation, crystallization, and optionally formulation.

10 Solubilization means the dissolution of derivatized protein and protein sufficiently to allow them to form hexamers. Hexamerization refers to the process wherein molecules of protein and derivatized protein bind with zinc(II) atoms to form hexamers. Complexation denotes 15 the formation of insoluble complexes between the hexamers and protamine. Precipitation results typically from the formation of insoluble complexes. Crystallization involves the conversion of precipitated hexamer/protamine complexes into crystals, typically, rod-like crystals.

20 Solubilization is carried out by dissolving the derivatized protein and protein in an aqueous solvent. The aqueous solvent may be, for example, an acidic solution, a neutral solution, or a basic solution. The aqueous solvent may be comprised partially of a miscible organic solvent, 25 such as ethanol, acetonitrile, dimethylsulfoxide, and the like. Acidic solutions may be, for example, solutions of HCl, advantageously from about 0.01 N HCl to about 1.0 N HCl. Other acids that are pharmaceutically acceptable may be employed as well. Basic solutions may be, for example, 30 solutions of NaOH, advantageously from about 0.01 N NaOH to about 1.0 N NaOH, or higher. Other bases that are pharmaceutically acceptable may be employed as well. For the sake of protein stability, the concentration of acid or

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base is preferably as low as possible while still being effective to adequately dissolve the protein and derivatized protein.

Most proteins (insulin and insulin analogs) and 5 many derivatized proteins may be dissolved to suitable concentrations at neutral pH. Solutions to dissolve derivatized proteins at neutral pH may contain a buffer and optionally, one or more additional solutes such as salts, phenolic compounds, zinc, and isotonicity agents.

10 When hexamerization occurs before homogenization, two populations of homogenous hexamers are formed first, and then the populations are mixed, thereby forming mixed hexamers. When homogenization occurs first, hexamerization yields hybrid hexamers. As mentioned above, to prepare 15 insoluble compositions comprised of hybrid hexamers, protein and derivatized protein are homogenized under conditions favoring dissociation to monomer or dimer aggregation states prior to hexamerization with a divalent metal cation. To achieve the necessary dissociation, the protein and 20 derivatized protein may be mixed under strongly acidic or strongly basic conditions. The degree of dissociation, and therefore, homogenization is influenced by the solution conditions chosen for this step. Insulin and related proteins readily self-associate in a series of reactions 25 producing dimers, hexamers, and other associated forms. The distribution of these association forms at equilibrium is dependent on many parameters, including pH. These association reactions are commonly thought to involve primarily monomer-dimer-hexamer assembly. Consequently, 30 depending on the solution conditions chosen, homogenization should accomplish the mixing of monomers, dimers, or a mixture thereof. Homogenization in 1 N HCl, for example, could involve a higher fraction of monomer mixing than in

0.1 N HCl, which would probably involve more dimer mixing. For the preparation of compositions comprised of hybrid hexamers, the homogenization process will be effective provided that only a very small or negligible fraction of 5 homogeneous hexamers of the protein or derivatized protein exist under the homogenization conditions employed.

Compositions comprised of mixed hexamers incorporate predominantly two types of hexamers, namely hexamers of the protein, and hexamers of the derivatized 10 protein. In this case, the homogenization step occurs after the hexamerization step, and achieves the homogenization of the hexamers prior to complexation with the complexing compound. Consequently, the homogenization step is performed under solution conditions that stabilize the 15 Zn(II)-insulin hexamer. Solution conditions that stabilize insulin hexamers are well known in the literature.

The solution conditions required for hexamerization are those that allow the formation of the hybrid hexamers or mixed hexamers in solution. These 20 conditions will be identical or very similar to the conditions under which insulin or insulin analogs are made to hexamerize. Typically, hexamerization requires zinc and a neutral to slightly basic pH, which is taken to be from about pH 6.8 to about pH 8.4. The presence of a hexamer-25 stabilizing compound advantageously influences hexamerization by promoting the R6 or the T3R3 conformations of the derivatized protein, and in certain instances, of the protein also. For certain monomeric insulin analogs, a hexamer-stabilizing compound is required to form hexamers.

30 For compositions comprised of hybrid hexamers, seven hexameric species are expected: P₆, P₅D₁, P₄D₂, P₃D₃, P₂D₄, P₁D₅, and D₆, where P represents the protein monomer,

and D represents the derivatized protein monomer. The statistical distribution of hexamers is expected to conform to a Poisson distribution, and will be influenced by the relative proportion of protein and derivatized protein, and 5 by the degree of dissociation prior to hexamerization. For example, from a homogenized solution constituted predominantly of dimers, four major hybrid hexamer species are expected: P_6 , P_4D_2 , P_2D_4 , and D_6 . For compositions comprised of mixed hexamers, only two hexameric species are 10 expected to predominate: P_6 and D_6 .

The complexation step must involve the combination a complexing compound with hexamer under solution conditions where each is initially soluble. This could be accomplished by combining separate solutions of hexamers and of 15 protamine, or by first forming a solution of protein, derivatized protein, and protamine at acidic or basic pH, and then shifting the pH to the neutral range.

During crystallization, the solution conditions must stabilize the crystallizing species, and promote the 20 conversion of precipitate to solute to crystal. Thus, the solution conditions will determine the rate and outcome of crystallization. Crystallization likely involves a complex equilibrium involving non-crystalline precipitate, dissolved hexamer-protamine complexes, and crystal. To obtain 25 microcrystals, the conditions chosen for crystallization must drive the equilibrium toward crystal formation. Also, in light of the hypothesized equilibrium, the solubility of the derivatized protein is expected to profoundly affect crystallization rate and size because lower solubility will 30 likely slow the net conversion from precipitate to solute to crystal. Furthermore, it is well-recognized that slowing the rate of crystallization often results in larger

crystals. Thus, the crystallization rate and crystal size are thought to depend on the size and nature of the derivatizing moiety on the derivatized protein.

Crystallization parameters that had been 5 previously thought to influence the crystallization rate and the size of crystals of the present invention are (see Brader I and Brader II): acyl group size and nature; temperature; the presence and concentration of "competing compounds" that compete with the protein and derivatized 10 protein for zinc, such as citrate, phosphate, and the like; the nature and concentration of phenolic compound(s); zinc concentration; the presence and concentration of a miscible organic solvent; the time permitted for crystallization; the pH and ionic strength; buffer identity and concentration; 15 the concentration of precipitants; the presence of seeding materials; the shape and material of the container; the stirring rate; and the total protein concentration. Temperature and the concentration of competing compounds were thought to be of particular importance.

20 It has now been discovered quite surprisingly that chloride ions, apart from their influence on ionic strength, play a significant role in determining the size of crystals and co-crystals. The exact nature of chloride's effect on crystal and co-crystal size is not known. A specific link 25 between chloride ion concentration and the rate of crystallization has not been previously described for these crystals and co-crystals.

Competing compounds, such as citrate, may affect 30 the rate at which crystals form, and indirectly, crystal size and quality. These compounds may exert their effect by forming coordination complexes with zinc in solution, thus competing with the relatively weak zinc binding sites on the surface of the hexamer for zinc. Occupation of these weak

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surface binding sites probably impedes crystallization. Additionally, many derivatized proteins are partially insoluble in the presence of little more than 0.333 zinc per mole of derivatized protein, and the presence of competing 5 compounds restores solubility, and permits crystallization. The optimum concentration of competing compound can be determined using routine techniques for any combination of protein and derivatized protein. As an upper limit, of course, is the concentration at which zinc is precipitated 10 by the competing compound, or the concentration at which residual competing compound would be pharmaceutically unacceptable, such as, when it would cause pain or irritation at the site of administration.

An example of a process for preparing the 15 precipitates and crystals of the present invention follows. A measured amount of the derivatized protein and a measured amount of the protein are dissolved in, or are combined to form a solution in an aqueous solvent containing a hexamer-stabilizing compound, such as a phenolic compound. To this 20 solution is added a solution of zinc as one of its soluble salts, for example $Zn(II)Cl_2$, to provide from about 0.3 moles of zinc per mole of derivatized insulin to about 0.7 moles, or to as much as 1.0 moles, of zinc per mole of total protein (protein + derivatized protein). Absolute ethanol, 25 or another miscible organic solvent, may optionally be added to this solution in an amount to make the solution from about 5% to about 10% by volume organic solvent. This solution may then be filtered through a 0.22 micron, low-protein binding filter. A protamine solution is prepared by 30 dissolving a measured amount of protamine in an aqueous solvent. This solution may be filtered through a 0.22 micron, low-protein binding filter. The solution of protein and derivatized protein and the protamine solution are

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combined, whereupon a precipitate forms initially. The resulting suspension is stirred slowly at room temperature (typically about 20-25°C), whereupon microcrystals are formed within a period from about 4 hours to about 10 days.

5 The microcrystals may then be separated from the mother liquor and introduced into a different solvent, for storage and administration to a patient. Examples of appropriate aqueous solvents are as follows: water for injection containing 25 mM TRIS, 5 mg/mL phenol and 16 mg/mL glycerol; water for injection containing 2 mg/mL sodium phosphate dibasic, 1.6 mg/mL m-cresol, 0.65 mg/mL phenol, and 16 mg/mL glycerol; and water for injection containing 25 mM TRIS, 5 mg/mL phenol, 0.1 M trisodium citrate, and 16 mg/mL glycerol.

15 In another process for preparing the insoluble compositions of the present invention, for example, a measured mass of dry derivatized protein and a measured mass of dry protein are dissolved together in an acidic aqueous solvent, such as 0.1 N - 1.0 N HCl. This solution is
20 stirred to insure thorough mixing of derivatized protein and protein. The ratio of derivatized protein powder to protein powder in this mixture is predefined to achieve a similar ratio of derivatized protein to protein in the insoluble composition to be produced. A separately prepared aqueous
25 solution comprised of a phenolic preservative and, optionally, a pharmaceutically acceptable buffer, is combined with the acidic solution of the proteins. The pH of the resulting solution is then adjusted to about 6.8 to about 8.4, preferably from about 6.8 to about 8.0, or
30 preferably to a pH of from about 7.2 to about 7.8, and most preferably from about 7.4 to about 7.8. To this solution is added a solution of zinc as one of its soluble salts, for example Zn(II)Cl₂, to provide from about 0.3 moles of zinc

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per mole of total insulin to about 4 moles of zinc per mole of total insulin. This solution is adjusted to a pH as given above, and preferably to about 7.4 -7.6, and may then be filtered through a 0.22 micron, low-protein binding 5 filter. A solution of protamine is prepared by dissolving a measured mass of protamine in an aqueous solvent. The protamine solution may be filtered through a 0.22 micron, low-protein binding filter. The solution of protein and derivatized protein and the protamine solution are combined, 10 whereupon a precipitate forms initially. The resulting suspension is stirred slowly at room temperature (typically about 20-25°C), whereupon microcrystals are formed within a period from about 4 hours to about 10 days.

In another process for preparing the insoluble 15 compositions of the present invention, a measured amount of a derivatized protein is first dissolved in an aqueous solvent containing a phenolic preservative. To this solution is added a solution of zinc as one of its soluble salts, for example $Zn(II)Cl_2$, to provide from about 0.3 20 moles of zinc per mole of derivatized protein to about 4 moles of zinc per mole of derivatized protein. The pH of the resulting solution is then adjusted to about 6.8 to about 8.4, preferably from about 6.8 to about 8.0, or preferably to a pH of from about 7.2 to about 7.8, and most 25 preferably from about 7.4 to about 7.8. A second solution is prepared separately wherein a measured amount of a protein selected from the group consisting of insulin, insulin analogs, and proinsulin is dissolved in an aqueous solvent containing a phenolic preservative. To this 30 solution is added a solution of zinc as one of its soluble salts, for example $Zn(II)Cl_2$, to provide from about 0.3 moles of zinc per mole of protein to about 4 moles of zinc

per mole of protein. The pH of the resulting solution is then adjusted to about 6.8 to about 8.4, preferably from about 6.8 to about 8.0, or preferably to a pH of from about 7.2 to about 7.8, and most preferably from about 7.4 to 5 about 7.8, or 7.4 - 7.6. Portions of the derivatized protein solution and the protein solution are combined in a ratio that is predefined in order to achieve a similar ratio of derivatized protein to protein in the insoluble composition. This solution is stirred to insure thorough 10 mixing of derivatized protein and protein. This solution is then adjusted to a pH of about 7.6, and may then be filtered through a 0.22 micron, low-protein binding filter. A protamine solution is prepared separately by dissolving a measured amount of protamine in an aqueous solvent. This 15 protamine solution may be filtered through a 0.22 micron, low-protein binding filter. The solution of protein and derivatized protein and the protamine solution are combined, whereupon a precipitate forms initially. The resulting suspension is stirred slowly at room temperature (typically 20 about 20-25°C), whereupon microcrystals are formed within a period from about 4 hours to about 10 days.

While not describing all of the very many types of processes that will produce the insoluble compositions of the present invention in any way, the following are yet 25 further processes of the present invention:

30 dissolving a protein, a derivatized protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent having a pH that will permit the formation of hexamers, and adding a complexing compound;

30 dissolving a protein, a derivatized protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent having a pH that will not permit the

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formation of hexamers, adjusting the pH to between about 6.8 and about 7.8, and adding a complexing compound;

5 dissolving a protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent having a pH that will permit the formation of hexamers, separately, dissolving a derivatized protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent having a pH that will permit the formation of hexamers, thoroughly mixing together these two solution,
10 and then adding a complexing compound;

dissolving a protein, a hexamer-stabilizing compound, a divalent metal cation, and a complexing compound in an aqueous solvent, wherein the resulting solution has a pH at which precipitation does not occur, separately,

15 dissolving a derivatized protein, a hexamer-stabilizing compound, a divalent metal cation, and a complexing compound in an aqueous solvent, wherein the resulting solution has a pH at which precipitation does not occur, thoroughly mixing together these two solutions, and adjusting the pH of the
20 solution to a value at which precipitation occurs;

dissolving a protein, a derivatized protein, a hexamer-stabilizing compound, a divalent metal cation, and a complexing compound in an aqueous solvent, wherein the resulting solution has a pH at which precipitation does not occur and adjusting the pH of the solution to a value at
25 which precipitation occurs;

dissolving a protein, a derivatized protein, a hexamer-stabilizing compound, and a divalent metal cation, in an aqueous solvent, wherein the resulting solution has a pH at which precipitation will not occur when a complexing agent is added, adding a complexing compound, and adjusting the pH of the solution to a value at which precipitation
30 occurs;

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dissolving a protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent, wherein the resulting solution has a pH at which precipitation will not occur when a complexing compound is

5 added, separately, dissolving a derivatized protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent, wherein the resulting solution has a pH at which precipitation will not occur when a complexing compound is added, thoroughly mixing together these two

10 solutions, adding complexing compound to the solution, and adjusting the pH to a value at which precipitation occurs;

dissolving a protein, a protein derivative, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent, wherein the resulting solution has a pH

15 at which precipitation will not occur when a complexing compound is added, adjusting the pH of the solution to a value at which precipitation will occur when a complexing compound is added, and adding a complexing compound to the solution;

20 dissolving a protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent, wherein the resulting solution has a pH at which precipitation will not occur when a complexing compound is added, separately, dissolving a derivatized protein, a hexamer-stabilizing compound, and a divalent metal cation in an aqueous solvent, wherein the resulting solution has a pH

25 at which precipitation will not occur when a complexing compound is added; thoroughly mixing together these two solutions, adjusting the pH of the solution to a value at

30 which precipitation will occur when a complexing compound is added, and adding a complexing compound to the solution;

In a preferred embodiment, the microcrystals are prepared in a manner that obviates the need to separate the

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microcrystals from the mother liquor. Thus, it is preferred that the mother liquor itself be suitable for administration to the patient, or that the mother liquor can be made suitable for administration by dilution with a suitable 5 diluent. The term diluent will be understood to mean a solution comprised of an aqueous solvent in which is dissolved various pharmaceutically acceptable excipients, including without limitation, a buffer, an isotonicity agent, zinc, a preservative, protamine, and the like.

10 In addition to the protein, derivatized protein, divalent cation, complexing compound, and hexamer-stabilizing compound, pharmaceutical compositions adapted for parenteral administration in accordance with the present invention may employ additional excipients and carriers such 15 as water miscible organic solvents such as glycerol, sesame oil, aqueous propylene glycol and the like. When present, such agents are usually used in an amount less than about 2.0% by weight based upon the final formulation. For further information on the variety of techniques using 20 conventional excipients or carriers for parenteral products, please see Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, PA, USA (1985), which is incorporated herein by reference.

In the broad practice of the present invention, it 25 is also contemplated that a formulation may contain a mixture of the microcrystals and a soluble fraction of a protein selected from insulin, derivatized insulin, insulin analogs, and derivatized insulin analogs. Examples of such pharmaceutical compositions include sterile, isotonic, 30 aqueous saline solutions of insulin, an insulin analog, a derivatized insulin, or a derivatized insulin analog, buffered with a pharmaceutically acceptable buffer and pyrogen-free. Preferred for the soluble phase are insulin

or a rapid-acting insulin analog, such as, LysB28,ProB29-human insulin, or AspB28-human insulin. Such mixtures are designed to provide a combination of meal-time control of glucose levels, which is provided by the soluble insulin, 5 and basal control of glucose levels, which is provided by the insoluble insulin. The ratio of total protein (protein plus derivatized protein) in the insoluble phase and total protein in the soluble phase is in the range of about 9:1 to about 1:9. A preferred range of this ratio is from about 10 9:1 to about 1:1, and more preferably, about 7:3. Other ratios are 1:1, and 3:7.

An effective dose of crystals or co-crystals for inhalation requires inhalation of from about 0.5 $\mu\text{g}/\text{kg}$ to about 200 $\mu\text{g}/\text{kg}$ total protein. Preferably the dose is about 15 5 $\mu\text{g}/\text{kg}$ to about 100 $\mu\text{g}/\text{kg}$, about 10 $\mu\text{g}/\text{kg}$ to about 100 $\mu\text{g}/\text{kg}$, about 20 $\mu\text{g}/\text{kg}$ to about 100 $\mu\text{g}/\text{kg}$, or about 30 $\mu\text{g}/\text{kg}$ to about 100 $\mu\text{g}/\text{kg}$. More preferably, the dose is from about 10 $\mu\text{g}/\text{kg}$ to about 60 $\mu\text{g}/\text{kg}$, 20 $\mu\text{g}/\text{kg}$ to about 60 $\mu\text{g}/\text{kg}$, or 30 $\mu\text{g}/\text{kg}$ to about 60 $\mu\text{g}/\text{kg}$. A therapeutically effective 20 amount can be determined by a knowledgeable practitioner, who will take into account factors including insulin protein level, the physical condition of the patient, the patient's pulmonary status, the potency and bioavailability of the proteins, whether the total proteins are administered 25 together with another insulin, such as a fast-acting, or meal-time insulin, or with other therapeutic agents, or other factors known to the medical practitioner. Effective starting therapy can include "titration" of the patient, that is, starting at a low dose, monitoring blood glucose 30 levels, and increasing the dose as required to achieve desired blood glucose levels.

According to the invention, crystals or co-crystals are delivered by inhalation to achieve advantageous slow uptake of insulin protein compared both to inhalation of non-derivatized insulin protein and inhalation of 5 derivatized, but soluble, protein. Administration by inhalation results in pharmacokinetics comparable to subcutaneous administration of crystalline insulins.

According to the present invention, crystals or co-crystals are delivered by any of a variety of inhalation 10 devices and methods known in the art for administration of insulin, or other proteins, by inhalation [Rubsamen, U.S. Patent No. 5,364,838, issued 15 November, 1994; Rubsamen, U.S. Patent No. 5,672,581, issued September 30, 1997; Platz, et al., WIPO publication No. WO96/32149, published October 15, 1996; Patton, et al., WIPO publication No. WO95/24183, published September 14, 1995; Johnson, et al., U.S. Patent No. 5,654,007, issued August 5, 1997; Goodman, et al., U.S. Patent No. 5,404,871, issued April 11, 1995; Rubsamen, et al., U.S. Patent No. 5,672,581, issued September 30, 1997; 20 Gonda, et al., U.S. Patent No. 5,743,250, issued April 28, 1998; Rubsamen, U.S. Patent No. 5,419,315, issued May 30, 1995; Rubsamen, et al., U.S. Patent No. 5,558,085, issued September 24, 1996; Gonda, et al., WIPO publication No. WO98/33480, published August 6, 1998; Rubsamen, U.S. Patent 25 No. 5,364,838, issued November 15, 1994; Laube, et al., U.S. Patent No. 5,320,094, issued June 14, 1994; Eljamal, et al. U.S. Patent No. 5,780,014, issued July 14, 1998; Backstrom, et al., U.S. Patent NO. 5,658,878, issued August 19, 1997; Backstrom, et al., 5,518,998, issued May 21, 1996; 30 Backstrom, et al., 5,506,203, issued April 9, 1996; Meezan, et al., U.S. Patent No. 5,661,130, issued August 26, 1997; Hodson, et al., U.S. Patent No. 5,655,523, issued August 12, 1997; Schultz, et al., U.S. Patent No. 5,645,051, issued

July 8, 1997; Eisele, et al., U.S. Patent No. 5,622,166, issued April 22, 1997; Mecikalski, et al., U.S. Patent No. 5,577,497, November 26, 1996; Mecikalski, et al., U.S. Patent No. 5,492,112, issued February 20, 1996; Williams, et al., U.S. Patent No. 5,327,883, issued July 12, 1994; Williams, U.S. Patent No. 5,277,195, issued January 11, 1994].

Included among the devices used to administer crystals and co-crystals according to the present invention are those well-known in the art, such as, metered dose inhalers, liquid nebulizers, dry powder inhalers, sprayers, thermal vaporizers, and the like, and those provided by developing technology, including the AERx® pulmonary drug delivery system being developed by Aradigm Corporation, the dry powder formulation and delivery devices being developed by Inhale Therapeutic Systems, Inc., and the Spiros® dry powder inhaler system being developed by Dura Pharmaceuticals, Inc. Other suitable technology includes electrohydrodynamic aerosolizers. The inhalation device should deliver small particles, e.g., less than about 10 μm MMAD, preferably about 1-5 μm MMAD, for good respirability, and more preferably in the range of about 1 to about 3 μm MMAD, and most preferably from about 2 to about 3 μm MMAD.

In addition, the inhalation device must be practical, in the sense of being easy to use, small enough to carry conveniently, capable of providing multiple doses, and durable. Some specific examples of commercially available inhalation devices suitable for the practice of this invention are Turbohaler (Astra), Rotahaler (Glaxo), Diskus (Glaxo), the Ultravent nebulizer (Mallinckrodt), the Acorn II nebulizer (Marquest Medical Products), the Ventolin metered dose inhaler (Glaxo), the Spinhaler powder inhaler

(Fisons), or the like. Both insulin and fatty acid-acylated insulin insulin can be advantageously delivered by a dry powder inhaler or a sprayer. There are several desirable features of a dry powder inhalation device for administering 5 crystals or co-crystals. For example, delivery by such inhalation devices is advantageously reliable, reproducible, and accurate.

As those skilled in the art will recognize, the nature and quantity of the pharmaceutical composition, and 10 the duration of administration of a single dose depend on the type of inhalation device employed. For some aerosol delivery systems, such as nebulizers, the frequency of administration and length of time for which the system is activated will depend mainly on the concentration of 15 crystals or co-crystals in the aerosol. For example, shorter periods of administration can be used at higher concentrations of crystals or co-crystals in the nebulizer solution. Devices such as metered dose inhalers can produce higher aerosol concentrations, and can be operated for 20 shorter periods to deliver the desired amount of crystals or co-crystals. Devices such as dry powder inhalers deliver active agent until a given charge of agent is expelled from the device. In this type of inhaler, the amount of crystals or co-crystals in a given quantity of the powder determines 25 the dose delivered in a single administration.

The particle size of the crystals and co-crystals delivered by the inhalation device determines the extent to which the particles are conveyed into the lower airways or alveoli, where deposition is most advantageous because of 30 the large surface area. Preferably, at least about 10% of the crystals or co-crystals are deposited in the lower lung, preferably about 10% to about 20%, or more. It is known that the maximum efficiency of pulmonary deposition for

mouth-breathing humans is obtained at about 2 μm to about 3 μm MMAD. Above about 5 μm MMAD, pulmonary deposition decreases substantially. Below about 1 μm MMAD pulmonary deposition decreases, and it becomes difficult to deliver 5 particles with sufficient mass to be therapeutically effective. Preferably, the crystals and co-crystals have a particle size less than about 10 μm , preferably in the range of about 1 μm to about 5 μm MMAD, and more preferably in the range of about 1 to about 3 μm MMAD, and most preferably 10 from about 2 to about 3 μm MMAD.

Dry powder generation typically employs a method such as a scraper blade or an air blast to generate particles from a solid formulation of fatty acid-acylated insulin protein. The particles are generally generated in a 15 container and then transported into the lung of a patient via a carrier air stream. Typically, in current dry powder inhalers, the force for breaking up the solid and air flow is provided solely by the patient's inhalation. One suitable dry powder inhaler is the Turbohaler manufactured 20 by Astra. Administration by dry powder inhaler is a preferred method for crystals or co-crystals.

Inhalation delivery of the crystals and co-crystals of the present invention can be accomplished using inhaler devices such as, but not limited to, jet nebulizers, 25 dry powder inhalers, ultrasonic nebulizers, piston pump, or piezoelectric nebulizers. The liquid solutions for the nebulizers might also contain agents such as, but not limited to, buffering agents, preservatives, or surfactants. Dry powder formulations might include spray dried powders 30 from solutions of sugars or polyols such as, but not limited to sucrose, lactose, dextrose, mannitol, trehalose, starch, as well as buffering agents.

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Formulations of crystals or co-crystals for administration from a dry powder inhaler typically include a finely divided dry powder of the crystals or co-crystals; preferably produced without resort to milling or other 5 mechanical operations. The powder can also include an un-derivatized insulin or insulin analog to provide relatively rapid onset, and short duration of action, a bulking agent, buffer, carrier, excipient, another additive, or the like. Additives can be included in a dry powder formulation of 10 crystals and co-crystals, for example, to dilute the powder as required for delivery from the particular powder inhaler, to facilitate processing of the formulation, to provide advantageous powder properties to the formulation, to facilitate dispersion of the powder from the inhalation 15 device, to stabilize the formulation (e.g., antioxidants or buffers), to provide taste to the formulation, or the like.

Advantageously, the additive does not adversely affect the patient's airways. The crystals or co-crystals can be mixed with an additive so that the solid formulation 20 includes crystal or co-crystal particles mixed with or coated on particles of the additive. Typical additives include mono-, di-, and polysaccharides; sugar alcohols and other polyols, such as, for example, lactose, glucose, raffinose, melezitose, lactitol, maltitol, trehalose, 25 sucrose, mannitol, starch, or combinations thereof; surfactants, such as sorbitols, diphosphatidyl choline, or lecithin; or the like. Typically an additive, such as a bulking agent, is present in an amount effective for a purpose described above, often at about 50% to about 90% by 30 weight of the formulation. Additional agents known in the art for formulation of a protein can also be included in the formulation. See, for example, Japanese Patent No.

J04041421, published February 12, 1992 (Taisho Pharmaceutical).

Advantageously for administration as a dry powder, the crystals or co-crystals have an MMAD of less than about 5 10 microns, preferably about 1 to about 5 microns, and more preferably in the range of about 1 to about 3 μm MMAD, and most preferably, from about 2 to about 3 μm MMAD. The preferred particle size is effective for efficient delivery to and deposition in the alveoli of the patient's lung. 10 Preferably, the dry powder is largely composed of particles produced so that a majority of the particles have a size in the desired range.

A spray including crystals or co-crystals can be produced by forcing a suspension of crystals or co-crystals 15 through a nozzle under pressure. The nozzle size and configuration, the applied pressure, and the liquid feed rate can be chosen to achieve the desired output and particle size. An electrospray can be produced by an electric field in connection with a capillary or nozzle 20 feed. Advantageously, particles delivered by a sprayer have a particle size less than about 10 μm , preferably in the range of about 1 μm to about 5 μm MMAD, and more preferably in the range of about 1 to about 3 μm MMAD, and most 25 preferably from about 2 to about 3 μm MMAD. Administration as a spray is a preferred method for crystals and co-crystals.

Formulations of crystals and co-crystals suitable for use with a sprayer typically include crystals or co-crystals in an aqueous solution at a concentration of about 30 1 mg to about 20 mg of total protein per mL of solution. The formulation can include agents such as an excipient, a buffer, an isotonicity agent, a preservative, a surfactant,

and zinc. The formulation can also include an excipient or agent for stabilization of the protein, such as a buffer, a reducing agent, a bulk protein, or a carbohydrate. Bulk proteins useful in formulating crystals or co-crystals 5 include albumin, protamine, or the like. Typical carbohydrates useful in spray formulations include sucrose, mannitol, lactose, trehalose, glucose, or the like. The spray formulation can also include a surfactant, which can reduce or prevent surface-induced aggregation of the 10 crystals or co-crystals caused by atomization of the solution in forming an aerosol. Various conventional surfactants can be employed, such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitol fatty acid esters. Amounts will generally range between 0.001% 15 and 4% by weight of the formulation. Especially preferred surfactants for purposes of this invention are polyoxyethylene sorbitan monooleate, polysorbate 80, polysorbate 20, or the like. Additional agents known in the art for formulation of a protein also be included in the 20 formulation.

Crystals or co-crystals can be administered by a nebulizer, such as jet nebulizer or an ultrasonic nebulizer. Typically, in a jet nebulizer, a compressed air source is used to create a high-velocity air jet through an orifice. 25 As the gas expands beyond the nozzle, a low-pressure region is created, which draws a suspension of crystals or co-crystals through a capillary tube connected to a liquid reservoir. The suspension streaming from the capillary tube is sheared into unstable filaments and droplets as it exits 30 the tube, creating an aerosol. A range of configurations, flow rates, and baffle types can be employed to achieve the desired performance characteristics from a given jet nebulizer. In an ultrasonic nebulizer, high-frequency

electrical energy is used to create vibrational, mechanical energy, typically by employing a piezoelectric transducer. This energy is transmitted to the suspension of crystals or co-crystals either directly or through a coupling fluid, 5 creating an aerosol. Advantageously, particles containing crystals or co-crystals delivered by a nebulizer have a particle size less than about 10 μm , preferably in the range of about 1 μm to about 5 μm MMAD, and more preferably in the 10 range of about 1 to about 3 μm MMAD, and most preferably, from about 2 to about 3 μm MMAD.

Formulations of crystals suitable for use with a nebulizer, either jet or ultrasonic, typically include crystals or co-crystals in an aqueous solution at a concentration of about 1 mg to about 20 mg of total protein 15 per mL of solution. The formulation can include agents such as an excipient, a buffer, an isotonicity agent, a preservative, a surfactant, and, preferably, zinc. The formulation can also include an excipient or agent for stabilization of the proteins, such as a buffer, a reducing 20 agent, a bulk protein, or a carbohydrate. Bulk proteins useful in formulating include albumin, protamine, or the like. Typical carbohydrates useful in formulating include sucrose, mannitol, lactose, trehalose, glucose, or the like. The formulation can also include a surfactant, which can 25 reduce or prevent surface-induced aggregation of the fatty acid-acylated insulin protein caused by atomization of the solution in forming an aerosol. Various conventional surfactants can be employed, such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbital fatty 30 acid esters. Amounts will generally range between 0.001 and 4% by weight of the formulation. Especially preferred surfactants for purposes of this invention are

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polyoxyethylene sorbitan monooleate, polysorbate 80, polysorbate 20, or the like.

In a metered dose inhaler (MDI), a propellant, suspension of crystals or co-crystals, and any excipients or 5 other additives are contained in a canister as a mixture including a liquefied compressed gas. Actuation of the metering valve releases the mixture as an aerosol, preferably with a MMAD in the range of less than about 10 μm , preferably about 1 μm to about 5 μm , and more preferably 10 in the range of about 1 to about 3 μm MMAD, and, most preferably from about 2 to about 3 μm MMAD. The desired aerosol particle size can be obtained by employing a formulation of crystals or co-crystals produced by various methods known to those of skill in the art, including jet-milling, spray drying, critical point condensation, or the like. Preferably, mechanical methods are avoided by 15 controlled crystallization according to the present processes. Preferred metered dose inhalers include those manufactured by 3M or Glaxo and employing a 20 hydrofluorocarbon propellant.

Formulations of crystals or co-crystals for use with a metered-dose inhaler device will include the crystals or co-crystals as a finely divided powder, in a suspension in a non-aqueous medium, for example, suspended in a 25 propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, 30 dichlorotetrafluoroethanol and 1,1,1,2-tetrafluoroethane, HFA-134a (hydrofluroalkane-134a), HFA-227 (hydrofluroalkane-227), or the like. Preferably the propellant is a hydrofluorocarbon. The surfactant can be chosen to

stabilize the crystals or co-crystals as a suspension in the propellant, to protect the active agent against chemical degradation, and the like. Suitable surfactants include sorbitan trioleate, soya lecithin, oleic acid, or the like.

5 Additional agents or excipients can also be included in the formulation.

The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of 10 the invention, and are not intended as limiting the scope of the invention.

Particle sizes are determined as follows. The following equipment may be used to determine particle diameters for the crystals and co-crystals: Coulter® Multisizer 646 or 15 equivalent (Coulter Corporation, Hialeah, FL), a Sampling Stand II, Model 999 or equivalent (Coulter Corporation, Hialeah, FL), a 50 μm Coulter® aperture tube, a pH meter, calibrated with pH buffers that bracket the desired pH value.

20 A stock diluent (at 2 X concentration) is prepared containing in one liter of water, 7.56 g of dibasic sodium phosphate crystals, 3.2 g of m-cresol, 32 g of glycerin and 1.46 g of phenol. The pH is adjusted to 7.35-7.45 with 5N HCl or 5N NaOH, and the diluent is filtered using a 0.22 μm 25 or smaller pore size filter. Store at room temperature. A working diluent (at 1X concentration) is prepared by combining one volume of the stock diluent with one volume of water, and again filtering.

The sample to be tested is resuspended. If in a 30 vial, resuspend by 10 palm rolls and 10 inversions. If in a cartridge, resuspend by three cycles of 10 palm rolls and 10 inversions. Pipette 0.25 mL of the sample into 100 mL of working diluent. This generally gives a particle count in

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the range of 90,000 to 300,000 counts over 50 second sampling time. If the particle count is less than this range, the sample might be too dilute so a volume greater than 0.25 mL sample will have to be used to get the 5 appropriate number of counts. Add sufficient volume of the suspension to give a particle count in the range of 100,000 to 300,000. The coincidence correction should not exceed 15%. For more concentrated samples, a volume less than 0.25 mL may need to be used. Place the beaker containing this 10 diluted sample on the sampling stand making sure that the outer electrode is submerged. Perform one measurement per sample. Measurements are made with continuous, slow stirring over a sampling time of 50 seconds.

The following parameters are typical for the 15 instrument:

Page One

Orifice Diameter: 50 μm

Orifice Length: 53 μm

Set-up: Manual

20 Analysis: Sample

Calibration: Recall

Kd: 505.00 (default value) will vary according to each calibration.

Size: 5

25 Units: μm

Page Two

Current and Gain: Manual

Aperture Current: 400 μA

30 Gain: 4

Polarity: +

Instrument control: Time

Time: 50 s

Channel count: 0

35 Total count: 0t

Page Three

Channels: 256

Autoscaling: On

40 Edit: Off

Coincidence correction: On

Analytical volume:

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Particle relative density: 1
Diff values: %
End tone: On

5 Page Four
 Communications Set-up

10 RS232C Serial output
 Baud rate: 9600
 Device: Computer
 Autoplot: No

15 Print/Plot Options
 Channel Data: No
 Analog Plot: No
 Screen Dump: Yes
 Overlay Mode: No
 Format: Enhanced

20 Computer Options
 Send STX/ETX: No
 End Field Char: 59
 End Line Char(s): CR
 Loading Zeros: No

25 The Coulter® Multisizer is used for particle characterization, namely, particle number and size determination. This instrument operates on the principle that when a particle suspended in a conductive liquid passes through a small orifice having electrodes on either side, a change in electrical resistance occurs. The change in resistance is related to the particle volume, and causes a short electrical pulse that is essentially proportional to the particle volume. The measurement of particle volume allows calculation of the equivalent volume diameter. The 30 series of pulses is electronically sorted to produce a size distribution curve. The software associated with the instrument provides the number and volume statistics and 35 their distributions.

40 Abbreviations used in the following Preparations and Examples are as follows:

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BHI: biosynthetic human insulin, which is human insulin synthesized biosynthetically in an organism transformed with recombinant DNA

5 Zn-BHI: zinc crystals of biosynthetic human insulin, containing approximately 0.33 zinc atoms per molecule of human insulin

C6-BHI: B29-N^ε-hexanoyl-human insulin

C8-BHI: B29-N^ε-octanoyl-human insulin

C10-BHI: B29-N^ε-decanoyl-human insulin

10 VMSED: volume mean spherical equivalent diameter of the particle size distribution of crystals or co-crystals; units are microns

S.D.: standard deviation of the particle size distribution of crystals or co-crystals; units are microns

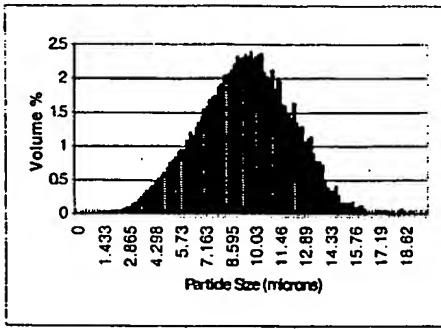
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Preparation 1

A stock solution of 40 mg/mL C8-BHI was prepared by dissolving lyophilized C8-BHI powder at pH 1.2. To 1 mL of 40 mg/mL C8-BHI was added 25 μ L 12.44 mg/mL zinc oxide. To 1 mL of this solution was added 4 mL of crystallization buffer containing 40 mg/mL glycerin, 4.4 mg/mL m-cresol, 1.8 mg/mL phenol, 9.375 mg/mL dibasic phosphate and 7.35 mg/mL trisodium citrate. The pH of the resulting solution was adjusted with 5N NaOH to 7.6. The solution was filtered with a Millipore Millex-GV filter and mixed with an equal volume of 0.64 mg/mL protamine sulfate. A precipitate formed immediately. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, some rod-shaped crystals were observed with some amorphous material.

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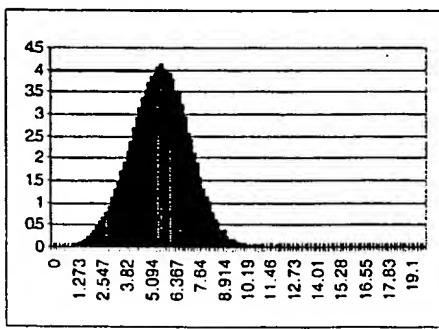
The particle size distribution was broad, with a volume mean spherical equivalent diameter (VMSED) of 9.7 microns.



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Preparation 2

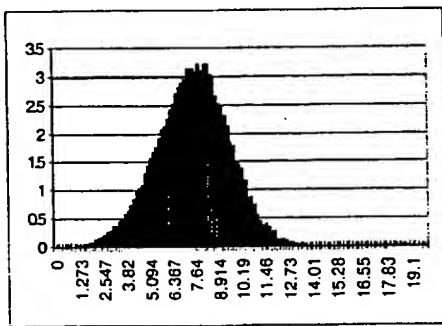
The procedure of Preparation 1 was carried out, except that the crystallization buffer contained 7.3 mg/mL sodium chloride. A precipitate formed immediately after adding the protamine sulfate. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate converted to rod-shaped crystalline material. The particle size distribution was much narrower than in the absence of added sodium chloride, with a VMSED of 5.8 microns. This also provides a means to 15 prepare 100% C8-BHI/protamine crystals.



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Preparation 3

Stock solutions of C8-BHI and Zn-BHI both at 40 mg/mL were prepared at pH 1.2. 0.85 mL of 40 mg/mL C8-BHI was mixed with 0.15 mL of 40 mg/mL Zn-BHI. To this insulin 5 solution was added 21.25 μ L 12.44 mg/mL zinc oxide. To 1 mL of this solution was added 4 mL of crystallization buffer containing 40 mg/mL glycerin, 4.4 mg/mL m-cresol, 1.8 mg/mL phenol, 9.375 mg/mL dibasic phosphate and 7.35 mg/mL trisodium citrate. The pH of the resulting solution was 10 adjusted with 5N NaOH to 7.6. The solution was filtered with Millipore Millex-GV filter and mixed with an equal volume of 0.64 mg/mL protamine sulfate. A precipitate formed immediately. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the 15 amorphous precipitate converted to rod-shaped crystalline material. The VMSED was 7.7 microns.



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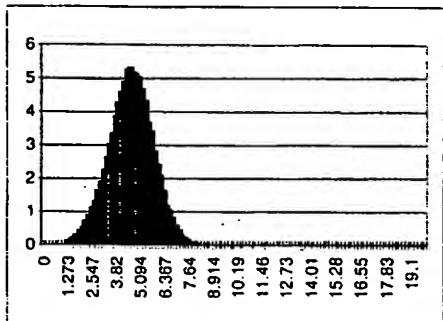
Preparation 4

The procedure of Preparation 3 was carried out, except that the crystallization buffer contained 7.3 mg/mL sodium chloride. A precipitate formed immediately after adding the protamine sulfate. The sample was stored 25 undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate converted to rod-shaped

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crystalline material. The effect of adding sodium chloride was clear and significant. Again the particle size distribution was much narrower and the VMSED was reduced to 4.8 microns.

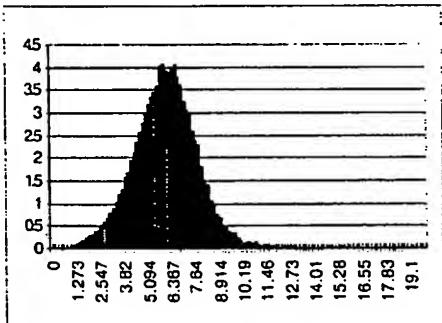
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Preparation 5

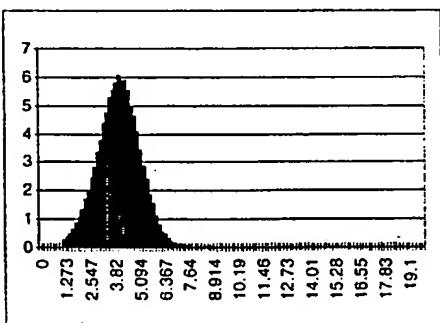
Stock solutions of C8-BHI and Zn-BHI both at 40 mg/mL were prepared at pH 1.2. 0.75 mL of 40 mg/mL C8-BHI was mixed with 0.25 mL of 40 mg/mL Zn-BHI. To this insulin solution was added 18.75 μ L 12.44 mg/mL zinc oxide. To 1 mL of this solution was added 4 mL of crystallization buffer containing 40 mg/mL glycerin, 4.4 mg/mL m-cresol, 1.8 mg/mL phenol, 9.375 mg/mL dibasic phosphate and 7.35 mg/mL trisodium citrate. The pH of the resulting solution was adjusted with 5N NaOH to 7.6. The solution was filtered with Millipore Millex-GV filter and mixed with an equal volume of 0.64 mg/mL protamine sulfate. A precipitate formed immediately. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate converted to rod-shaped crystalline material. The VMSED was 6.2 microns.

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Preparation 6

The procedure of Preparation 5 was carried out, except that the crystallization buffer contained 7.3 mg/mL sodium chloride. A precipitate formed immediately after adding the protamine sulfate. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate converted to rod-shaped crystalline material. The effect of adding sodium chloride was clear and significant. Again the particle size distribution was much narrower and the VMSED was reduced to 4.2 microns.



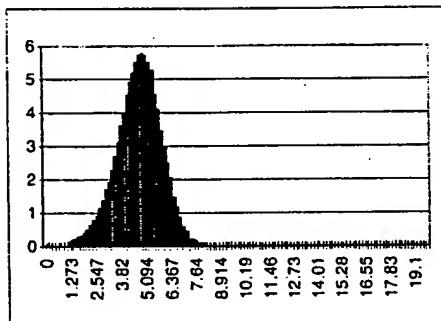
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Preparation 7

Stock solutions of C8-BHI and Zn-BHI both at 40 mg/mL were prepared at pH 1.2. 0.65 mL of 40 mg/mL C8-BHI was mixed with 0.35 mL of 40 mg/mL Zn-BHI. To this insulin solution was added 16.25 μ L 12.44 mg/mL zinc oxide. To 1 mL of this solution was added 4 mL of crystallization buffer

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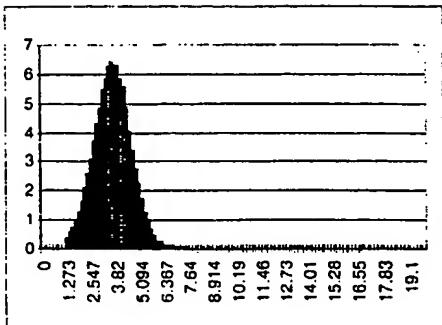
containing 40 mg/mL glycerin, 4.4 mg/mL m-cresol, 1.8 mg/mL phenol, 9.375 mg/mL dibasic phosphate and 7.35 mg/mL trisodium citrate. The pH of the resulting solution was adjusted with 5N NaOH to 7.6. The solution was filtered 5 with Millipore Millex-GV filter and mixed with equal volume of 0.64 mg/mL protamine sulfate. A precipitate formed immediately. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate had converted to rod shaped 10 crystalline material. The VMSED was 5.0 microns.



Preparation 8

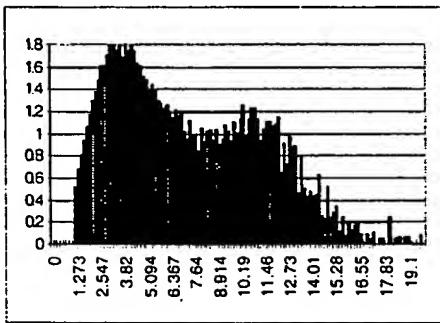
The procedure of Preparation 7 was carried out, 15 except that the crystallization buffer contained 7.3 mg/mL sodium chloride. A precipitate formed immediately after adding the protamine sulfate. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate had converted to rod-shaped 20 crystalline material. The effect of adding sodium chloride was clear and significant. Again the particle size distribution was much narrower and the VMSED was reduced to 3.8 microns.

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Preparation 9

The procedure of Preparation 1 was carried out, except that the C8-BHI was initially dissolved at pH 2.4 instead of pH 1.2. After 24 hours, the material was still 5 largely amorphous with a few rod-shaped crystals observed. The particle size distribution was multi-modal.



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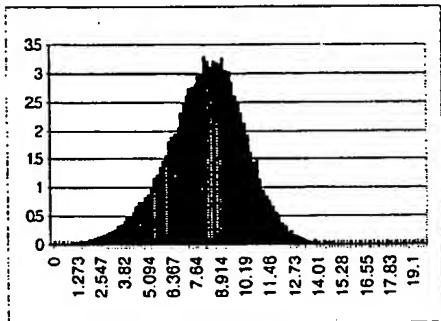
Preparation 10

The procedure of Preparation 9 was carried out, except that the crystallization buffer contained 7.3 mg/mL sodium chloride. A precipitate formed immediately after adding the protamine sulfate. The sample was stored 15 undisturbed at a controlled temperature of 25°C. After 24 hours, unlike the preparation without added sodium chloride, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 8.4 microns. This

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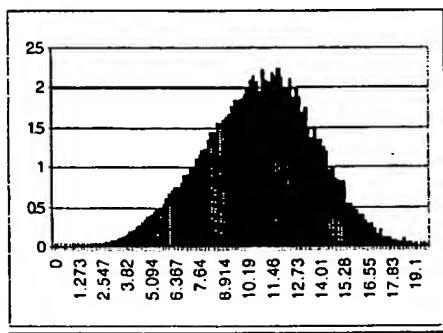
represented an example of controlling the preparation of 100% C8-BHI protamine crystals to achieve a Gaussian distribution having a narrower distribution of particle sized, and a lower mean.

5



Preparation 11

The procedure of Preparation 3 was carried out,
10 except that the C8-BHI was initially dissolved at pH 2.4 instead of pH 1.2. After 24 hours, the amorphous precipitate had converted to rod shaped crystalline material. The VMSED was 11.0 microns.



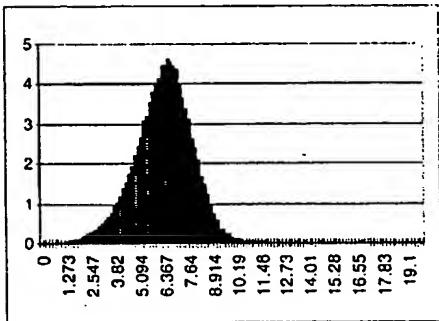
15

Preparation 12

The procedure of Preparation 11 was carried out, except that the crystallization buffer contained 7.3 mg/mL sodium chloride. A precipitate formed immediately after
20 adding the protamine sulfate. The sample was stored

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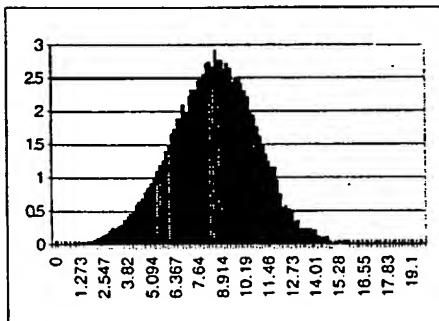
undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The effect of adding sodium chloride was clear and significant. Again the particle size distribution was much narrower and the VMSED was reduced to 6.5 microns.



10

Preparation 13

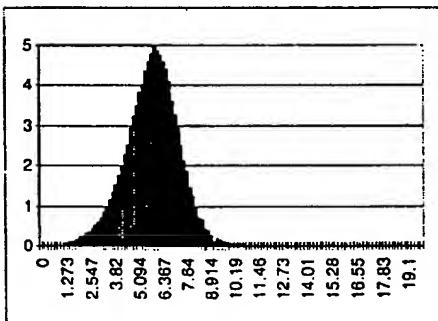
The procedure of Preparation 5 was carried out, except that the C8-BHI was initially dissolved at pH 2.4 instead of pH 1.2. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 8.7 microns.



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Preparation 14

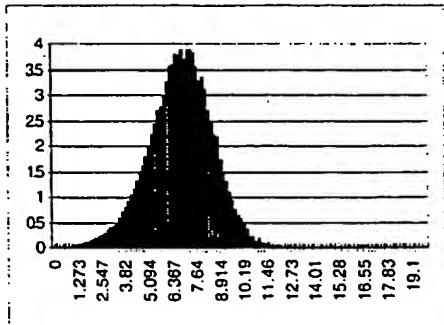
The procedure of Preparation 13 was carried out, except that the crystallization buffer contained 7.3 mg/mL sodium chloride. A precipitate formed immediately after 5 adding the protamine sulfate. The sample was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The effect of adding sodium chloride was clear and significant. Again the particle size 10 distribution was much narrower and the VMSED was reduced to 6.0 microns.



Preparation 15

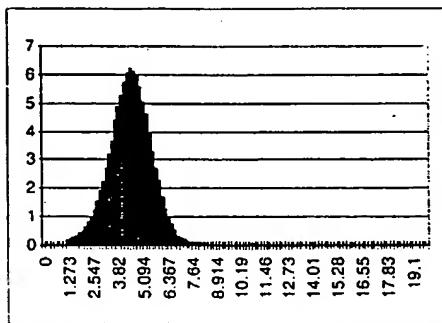
15 The procedure of Preparation 7 was carried out, except that the C8-BHI was initially dissolved at pH 2.4 instead of pH 1.2. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 7.0 microns.

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Preparation 16

The procedure of Preparation 15 was carried out,
5 except that the crystallization buffer contained 7.3 mg/mL
sodium chloride. A precipitate formed immediately after
adding the protamine sulfate. The sample was stored
undisturbed at a controlled temperature of 25°C. After 24
hours, the amorphous precipitate had converted to rod-shaped
10 crystalline material. The effect of adding sodium chloride
was clear and significant. Again the particle size
distribution was much narrower and the VMSED was reduced to
4.7 microns.



15

Preparation 17

A C8-BHI solution was prepared by dissolving 61.4 mg lyophilized powder of C8-BHI in 1.54 mL 0.1 N HCl. A Zn-BHI solution was prepared by dissolving 20.5 mg of Zn-BHI
20 crystals in 0.51 mL 0.1 N HCl. To the Zn-BHI solution was

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added 1.5 mL of the C8-BHI solution. To the Zn-BHI and C8-BHI solution was added 49 μ L 12.44 mg/mL Zinc oxide stock solution. To this solution was added 8 mL of crystallization buffer containing 40 mg/mL glycerin, 4.4 mg/mL m-cresol, 1.8 mg/mL phenol, 9.375 mg/mL dibasic phosphate and 7.35 mg/mL trisodium citrate. The pH of the resulting solution was adjusted with 5N NaOH to 7.6. The solution was filtered with Millipore Millex-GV filter and mixed with equal volume of 0.64 mg/mL protamine sulfate. A precipitate formed immediately. The suspension was equally divided and stored undisturbed at controlled temperatures of 20, 25 and 30°C. After 24 hours, the amorphous precipitates had in each sample converted to rod-shaped crystalline material. The effect of temperature was clear and significant. The VMSED was 7.8, 6.9 and 5.5 microns at 20, 25 and 30°C respectively.

Summary of Preparations 1 - 17

| Preparation | C8-BHI % | NaCl added | Dissolu-tion pH | Day 1 | | Day 30 | |
|-------------|----------|------------|-----------------|-------|------|--------|------|
| | | | | VMSED | S.D. | VMSED | S.D. |
| 1 | 100 | None | 1.2 | 9.65 | 2.68 | 9.60 | 2.58 |
| 2 | 100 | 50mM | 1.2 | 5.78 | 1.55 | 6.70 | 1.62 |
| 3 | 85 | None | 1.2 | 7.68 | 2.00 | 8.68 | 2.01 |
| 4 | 85 | 50mM | 1.2 | 4.79 | 1.20 | 5.72 | 1.28 |
| 5 | 75 | None | 1.2 | 6.24 | 1.69 | 7.08 | 1.62 |
| 6 | 75 | 50mM | 1.2 | 4.21 | 1.12 | 4.70 | 1.06 |
| 7 | 65 | None | 1.2 | 4.99 | 1.20 | 5.97 | 1.24 |
| 8 | 65 | 50mM | 1.2 | 3.85 | 1.21 | 4.45 | 1.23 |
| 9 | 100 | None | 2.4 | - | - | 10.91 | 6.61 |
| 10 | 100 | 50mM | 2.4 | 8.38 | 2.05 | 8.88 | 2.24 |
| 11 | 85 | None | 2.4 | 11.03 | 2.93 | 11.50 | 2.93 |
| 12 | 85 | 50mM | 2.4 | 6.52 | 1.53 | 7.25 | 1.50 |
| 13 | 75 | None | 2.4 | 8.73 | 2.31 | 9.35 | 2.21 |
| 14 | 75 | 50mM | 2.4 | 6.01 | 1.42 | 6.74 | 1.36 |
| 15 | 65 | None | 2.4 | 7.05 | 1.71 | 7.75 | 1.68 |
| 16 | 65 | 50mM | 2.4 | 4.67 | 1.14 | 5.30 | 1.20 |
| | | | Temp. | | | | |
| 17 | 75 | None | 20°C | 7.80 | 2.28 | - | - |
| 17 | 75 | None | 25°C | 6.93 | 1.84 | - | - |
| 17 | 75 | None | 30°C | 5.52 | 1.58 | - | - |

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The following conclusions are drawn on the basis of Preparations 1 - 17, above. First, at the same percentage of C8-BHI, pH 1.2 dissolution gives smaller and tighter particle size distribution than pH 2.4 dissolution.

5 Second, a lower percentage of C8-BHI gives smaller co-crystals and a smaller standard deviation. Third, adding sodium chloride also yields smaller co-crystals and a smaller standard deviation. This clearly provides a means to control the particle size with possible different

10 variations in formulations and manufacturing processes. Finally, adding sodium chloride also clearly provides a means to prepare 100% C8-BHI crystals.

Preparation 18

15 A 50 mL stock solution of C8-BHI at approximately 26.5 mg/mL and Zn-BHI at 8.9 mg/mL was prepared at pH 1.2. To this insulin solution was added approximately 1mL of 12.44 mg/mL zinc oxide. To this solution was added 200 mL of crystallization buffer containing 40 mg/mL glycerin, 4.4 mg/mL m-cresol, 1.8 mg/mL phenol, 9.375 mg/mL dibasic phosphate and 7.35 mg/mL trisodium citrate. The pH of the resulting solution was adjusted with 5N NaOH to 7.6. The solution was filtered with Millipore Millex-GV filter and mixed with equal volume of 0.64 mg/mL protamine sulfate. A 20 precipitate formed immediately. The suspension was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 7.8 microns.

30

Preparation 19

An approximately 120 mL stock solution of C8-BHI at approximately 10.5 mg/mL and approximately 3.5 mg/mL Zn-BHI was prepared at pH 2.4. To this insulin solution was

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added approximately 1 mL of 12.44 mg/mL zinc oxide. To this solution was added approximately 125 mL of crystallization buffer containing 64 mg/mL glycerin, 7.0 mg/mL m-cresol, 2.9 mg/mL liquefied phenol, 15 mg/mL dibasic sodium phosphate 5 and 11.76 mg/mL trisodium citrate. The pH of the resulting solution was adjusted with 5N NaOH to 7.6. Water was added to bring the volume to 250 mL. The solution was filtered with Millipore Millex-GV filter and mixed with an equal volume of 0.64 mg/mL protamine sulfate. A precipitate 10 formed immediately. The suspension was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 9.2 microns.

15

Preparation 20

The procedure of Preparation 19 was followed, except that the crystallization buffer also contained 11.69 mg/mL sodium chloride. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline 20 material. The VMSED was 5.3 microns.

Preparation 21

A 25 mL stock solution of C8-BHI at approximately 10.5 mg/mL and approximately 3.5 mg/mL Zn-BHI was prepared 25 at pH 2.5. To 2.5 mL of this insulin solution was added approximately 0.24 μ L of 12.44 mg/mL zinc oxide. To this solution was added approximately 2.5 mL of crystallization buffer containing 64 mg/mL glycerin, 7.0 mg/mL m-cresol, 2.9 mg/mL liquefied phenol, 15 mg/mL dibasic sodium phosphate 30 and 11.76 mg/mL trisodium citrate. The pH of the resulting solution was adjusted with 5N NaOH to 7.6. The solution was filtered with Millipore Millex-GV filter and mixed with equal volume of 0.64 mg/mL protamine sulfate. A precipitate

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formed immediately. The suspension was stored undisturbed at a controlled temperature of 25°C. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 7.09 microns.

5

Preparation 22

The procedure of Preparation 21 was followed, except that the crystallization buffer also contained 11.69 mg/mL sodium chloride. After 24 hours, the amorphous 10 precipitate had converted to rod-shaped crystalline material. The VMSED was 5.72 microns.

Preparation 23

The procedure of Preparation 21 was followed, 15 except that the crystallization buffer also contained 14.91 mg/mL potassium chloride. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 5.39 microns.

20

Preparation 24

The procedure of Preparation 21 was followed, except that the crystallization buffer also contained 16.41 25 mg/mL sodium acetate. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 7.39 microns.

Preparation 25

The procedure of Preparation 21 was followed, 30 except that the crystallization buffer also contained 46.02 mg/mL sodium tartrate. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 8.07 microns.

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Preparation 26

The procedure of Preparation 21 was followed, except that the crystallization buffer also contained 28.41 mg/mL sodium sulfate. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 7.07 microns.

Preparation 27

The procedure of Preparation 21 was followed, except that the crystallization buffer also contained 13.6 mg/mL sodium formate. After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material. The VMSED was 6.90 microns.

15

Summary of Preparations 18 - 27

| Preparation | Scale | Salt added (50 mM) | VMSED | SD |
|-------------|-------|-----------------------|-------|------|
| 18 | 500mL | None | 7.81 | 1.77 |
| 19 | 500mL | None | 9.24 | 2.46 |
| 20 | 500mL | NaCl | 5.31 | 1.32 |
| 21 | 10mL | None | 7.09 | 1.85 |
| 22 | 10mL | NaCl | 5.72 | 1.45 |
| 23 | 10mL | KCl | 5.39 | 1.40 |
| 24 | 10mL | Na acetate | 7.39 | 2.07 |
| 25 | 10mL | Na tartrate | 8.07 | 2.21 |
| 26 | 10mL | Na sulfate | 7.07 | 1.70 |
| 27 | 10mL | Na formate | 6.90 | 1.88 |

The following conclusions are drawn from
20 Preparations 18 - 27. First, the scale at which the crystallization is carried out affects the mean particle size only when no sodium chloride is added. The addition of sodium chloride, or just chloride anion, will permit more reproducible results at a larger scale, using a
25 manufacturable process. The effect of sodium chloride is due to chloride, not sodium. Other anions tested had little or no effect. These results also show that the effect of

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sodium chloride is not due to an increase in ionic strength, but rather to the specific effect of the chloride anion.

Preparation 28

5 **100% C8-BHI Crystal Formulations For Rat Intratracheal
Instillation Experiments**

A dry lyophilized powder of C8-BHI (471 mg) was dissolved in 11.77 mL of 0.1 N HCl. To this solution was 10 added 3.532 mL of a 1000 ppm solution of zinc nitrate and stirred. To this solution was added 47.08 mL of an aqueous solution comprised of 9.5 mg/mL disodium phosphate heptahydrate, 0.375 M NaCl, 4 mg/mL m-cresol, 1.65 mg/mL phenol, and 16 mg/mL glycerol at pH 7.63. The pH was 15 adjusted to 7.6 with small quantities of 1N HCl and 1N NaOH. This solution was then filtered through a 0.2 micron low protein binding filter. A second solution was prepared by dissolving 86.8 mg of a dry powder of protamine sulfate in 115.73 mL of water and then filtered through a 0.2 micron 20 low protein binding filter. 55.95 mL of C8-BHI solution was mixed with 55.95 mL of protamine sulfate solution. A white precipitate formed. This suspension was stirred gently to complete mixing. The preparation was allowed to stand 25 undisturbed for 24 hours in a 30°C water bath. Inspection under an optical microscope revealed the presence of microcrystalline solid comprising rod-like crystals. Measurement of particle size distribution by the Coulter technique revealed a VMSED of 4.7 microns.

The mother liquor of this preparation was 30 exchanged with an aqueous solution comprised of 0.7 mg/mL phenol, 1.6mg/mL m-cresol and 0.3 mg/mL of disodium phosphate heptahydrate by the following procedure. 50 mL of suspension was centrifuged at 3000 rpm for 12 minutes at 23°C, 40 mL of mother liquor was removed without disturbing

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the solid phase and replaced with the aqueous solution comprised of 0.7 mg/mL phenol, 1.6mg/mL m-cresol and 0.3 mg/mL of disodium phosphate heptahydrate. This procedure was repeated twice more. Following this exchange of mother 5 liquor, the microcrystals were again analyzed using the Coulter counter, and were found to have a VMSED of 3.5 microns.

Analytical characterization by HPLC revealed that almost all of the insulin and protamine sulfate was present 10 in the solid phase. For rat intratracheal instillation experiments, these formulations were diluted to the required concentration by appropriate dilution with an aqueous solution containing 0.7mg/mL phenol, 1.6mg/mL m-cresol and 0.3 mg/mL of disodium phosphate heptahydrate.

15

Preparation 29

75% C8-BHI Crystal Formulations For Rat Intratracheal Instillation Experiments

20 A dry lyophilized powder of C8-BHI (330 mg) was dissolved in 8.25 mL of 0.1 N HCl. A dry powder of human insulin-Zn crystals (116 mg) was dissolved in 2.9 mL of 0.1 N HCl. 2.75 mL of the later solution was mixed with C8-BHI solution to produce a mixture of C8-BHI and human insulin in 25 approximate weight ratio of 75:25. This solution was stirred to mix. To this solution was added 2.866 mL of a 1000 ppm solution of zinc nitrate and stirred. To this solution was added 47 mL of an aqueous solution comprised of 9.5 mg/mL disodium phosphate heptahydrate, 0.375 M NaCl, 4 mg/mL m-cresol, 1.65 mg/mL phenol, and 16 mg/mL glycerol at 30 pH 7.63. The pH was adjusted to 7.6 with small quantities of 1N HCl and 1N NaOH. This solution was then filtered through a 0.2 micron low protein binding filter. A second solution was prepared by dissolving 86.8 mg of a dry powder

of protamine sulfate in 115.73 mL of water and then filtered through a 0.2 micron low protein binding filter. 50mL of the solution of the mixture of C8-BHI and human insulin was mixed with 50 mL of protamine sulfate solution. A white precipitate formed. This suspension was stirred gently to complete mixing. The preparation was allowed to stand undisturbed for 24 hours at 30°C. Inspection under an optical microscope revealed the presence of microcrystalline solid comprising rod-like crystals. Measurement of particle size distribution by Coulter technique revealed a VMSED of 10 4.4 microns.

The mother liquor of this preparation was exchanged with an aqueous solution comprised of 0.7 mg/mL phenol, 1.6mg/mL m-cresol and 0.3 mg/mL of disodium 15 phosphate heptahydrate as described above in Preparation 28. Following the exchange of mother liquor, the microcrystals were determined to have VMSED of 3.9 microns.

Analytical characterization by HPLC revealed that almost all of the insulin and protamine sulfate was present 20 in the solid phase. For rat intratracheal instillation experiments, these formulations were diluted to the required concentration by appropriate dilution with an aqueous solution containing 0.7 mg/mL phenol, 1.6mg/mL m-cresol and 0.3 mg/mL of disodium phosphate heptahydrate.

25 The procedures described in Preparations 28 and 29 demonstrate that crystals comprised of a derivatized insulin, together with zinc and protamine, can be produced in a size that is preferred for optimizing deposition in the deep lung (preferably <5 microns, and more preferably <3 30 microns) when the crystallization conditions are properly controlled. The critical parameters of these preparations were the concentration of NaCl present during

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crystallization (150 mM) and the temperature during crystallization (30°C).

Preparation 30

5 The mother liquors of Preparation 28 and Preparation 30 were exchanged with water by the following procedure. 5mL of suspension was centrifuged at 3000 rpm for 12 minutes at 23°C. 4 mL of mother liquor was removed without disturbing the solid phase and replaced with water. 10 This procedure was repeated twice more. The resulting suspension was freeze dried. The solid obtained after freeze drying was reconstituted in 5 mL of water. The resulting suspension contained small microcrystals when examined under an optical microscope.

15

Preparation 31

The mother liquors of Preparation 28 and Preparation 29 were exchanged as described in Preparation 30, except that a solution of 0.9 g NaCl per 100 mL was used 20 to wash the crystals. After three washes, the resulting suspension was freeze dried. The solid obtained after freeze drying was reconstituted in 5mL of water. The resulting suspension contained small microcrystals when examined under an optical microscope.

25

The procedures described in Preparations 30 and 31 demonstrate that the microcrystals obtained by the processes of the present invention (i.e., for example, Preparations 28 and 29) can be freeze-dried without loss of their crystalline nature. Such microcrystalline powders can be 30 used in dry powder inhalers for pulmonary delivery. These results also demonstrate that these freeze-dried microcrystals can be mixed with water to form stable aqueous suspensions with minimal excipients. Such aqueous

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suspensions can be used in nebulizers for pulmonary delivery.

Preparation 32

5 After crystallization according to the procedure described for either one of Preparations 28 or 29, as determined by optical microscopy, the microcrystals are separated from the mother liquor and are recovered by conventional solid/liquid separation methods. The recovered 10 microcrystals are then resuspended in a solution comprised of a buffer (e.g., 0.3 mg/mL dibasic sodium phosphate heptahydrate), a anti-microbial preservative (e.g., 0.65 mg/mL phenol and/or 1.6 mg/mL m-cresol), and an isotonicity agent (e.g., 16 mg/mL glycerol or 9 mg/mL NaCl), and the pH 15 is adjusted to 6.8.

Preparation 33

After crystallization according to the procedure described for either one of Preparations 28 or 29, as 20 determined by optical microscopy, the microcrystals are separated from the mother liquor and are recovered by conventional solid/liquid separation methods. The recovered microcrystals are then resuspended according to Preparation 32, except that the resuspension solution contains no 25 antimicrobial preservative. Such a preparation might be more suitable when the absence of irritants or unneeded excipients is desired.

Preparation 34
Dissolution of 75% C8-BHI co-crystals having VMSED of 2.1
microns

5 A preparation of 75% C8-BHI cocrystals was made by a procedure similar to preparation 29, on a 10 mL scale. The crystals prepared by this procedure had a VMSED of 2.1 microns.

10 The in-vitro dissolution rate of these crystals was examined using a spectrophotometric dissolution assay. 3 mL of phosphate buffered saline was placed in a quartz cuvette with a small stir bar. The solution was stirred at a fixed rate. The absorbance was zeroed. Five microliters of uniformly suspended formulation was quickly suspended at 15 the bottom of the cuvette. One minute after the addition of the formulation, absorbance data at 305 nm was collected as a function of time. Change of absorbance was followed as a function of time.

20 The absorbance decreases as the scattering particles dissolve. The time for the absorbance to decrease by half of its full decrease is denoted by the parameter $t_{1/2}$. This parameter is useful for comparing the rates of dissolution of different crystalline preparations. The greater $t_{1/2}$, the slower is the dissolution.

25 In the dissolution test described above, $t_{1/2}$ for the co-crystals prepared according to Preparation 34 was determined to be 50 minutes. Under similar conditions of dissolution, $t_{1/2}$ for NPH-human insulin crystals was typically about 10 minutes or less. Thus, despite its 30 smaller VMSED and expected concomitant increase in surface area, the co-crystals of Preparation 34 dissolved significantly slower than NPH. This observation supports the conclusion that the smaller microcrystals of the present

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invention will provide more sustained release *in vivo* as compared with NPH-human insulin crystals.

5

Preparations 35-40

The procedures of Preparations 28 and 29 above were followed at the 10 mL scale to produce three samples of crystals each, with the following exceptions: the crystallization buffer for the three samples contained 10 either no sodium chloride, 75 mM sodium chloride, or 150 mM sodium chloride; and the temperature was controlled at 30°C. Very small particles, with approximately 2 micron mean particle diameter and a narrow size distribution were obtained for both 75% and 100% C8-BHI compositions when 150 15 mM NaCl was present during crystallization.

| Preparation | Composition | [NaCl] (mM) | VMSED (microns) | S.D. (Microns) |
|-------------|-------------|-------------|-----------------|----------------|
| 35 | 100% C8-BHI | 0 | 4.4 | 2.9 |
| 36 | 100% C8-BHI | 75 | 4.1 | 1.3 |
| 37 | 100% C8-BHI | 150 | 2.1 | 0.7 |
| 38 | 75% C8-BHI | 0 | 4.2 | 1.1 |
| 39 | 75% C8-BHI | 75 | 3.6 | 1.0 |
| 40 | 75% C8-BHI | 150 | 2.2 | 0.6 |

Preparations 41-44

20 A dry powder of C8-BHI (24.0 mg) was dissolved in 1.20 mL of 0.1 N HCl. A separate solution was prepared by dissolving a dry powder of human insulin zinc crystals (8.0 mg) in 0.400 mL of 0.1 N HCl. These two solutions were combined and mixed to produce 1.60 mL of a solution mixture 25 of human insulin and C8-BHI. To this solution was added 448 microliters of a 15.3 mM solution of zinc chloride with stirring. To this solution was added 6.4 mL an aqueous solvent composed of 10 mg/mL dibasic sodium phosphate heptahydrate, 7.5 mg/mL trisodium citrate dihydrate, 4 mg/mL 30 m-cresol, 2 mg/mL phenol, and 40 mg/mL glycerol at pH 7.59.

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The pH was adjusted to 7.61 with small quantities of 1 N HCl and 1 N NaOH. This solution was then filtered through a 0.22 micron low protein binding filter. A second solution was prepared by dissolving 72.1 mg of protamine sulfate in 5 96 mL of water and then filtering through a 0.22 micron low protein binding filter. An 8 mL volume of the insulin mixture solution was combined with 8 mL of the protamine sulfate solution. An amorphous precipitate formed. This suspension was stirred gently to complete mixing. The 10 preparation was divided into four volumes of 4 mL which were allowed to stand undisturbed at temperatures of 15 °C, 25 °C, 30 °C and 35 °C, respectively, for 90 hours. Inspection under an optical microscope (1000x) revealed that in each case the amorphous precipitate had converted to a 15 microcrystalline solid of uniform appearance, comprising single, rod-like crystals. The preparations corresponding to temperatures of 15 °C, 25 °C, 30 °C and 35 °C were designated Preparations 41, 42, 43, and 44 respectively.

Each sample was centrifuged at 3000 rpm for 12 20 minutes to sediment the crystals. For each Preparation, 3.2 mL of supernatant was decanted off and replaced with 3.2 mL of an aqueous diluent comprising 4 mg/mL dibasic sodium phosphate heptahydrate, 3 mg/mL trisodium citrate dihydrate, 0.8 mg/mL phenol, and 16 mg/mL glycerol at pH 7.61. This 25 diluent exchange process was repeated a second and a third time, except on the third occasion the 3.2 mL was replaced

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with 2.8 mL of the aforementioned aqueous diluent. The VMSED of these preparations are shown in the table below.

| Preparation | Crystallization Temperature (°C) | VMSED (microns) |
|-------------|----------------------------------|-----------------|
| 41 | 15 | 9.0 |
| 42 | 25 | 6.0 |
| 43 | 30 | 5.5 |
| 44 | 35 | 4.9 |

5

Preparations 45 - 48

A dry powder of C8-BHI (24.0 mg) was dissolved in 0.60 mL of 0.1 N HCl. A separate solution was prepared by dissolving a dry powder of human insulin zinc crystals (8.2 mg) in 0.20 mL of 0.1 N HCl. These two solutions were 10 combined and mixed to produce 0.80 mL of a solution mixture of human insulin and C8-BHI. To this solution was added 300 microliters of a 15.3 mM solution of zinc chloride with stirring. This solution was divided into four separate volumes of 0.275 mL. Four different crystallization buffers 15 were prepared, each of which contained 35 mM dibasic sodium phosphate heptahydrate, 4 mg/mL m-cresol, 1.6 mg/mL phenol, and 40 mg/mL glycerol at pH 7.6, and each of which differed in sodium citrate concentrations, which were 0 mM, 12.5 mM, 37.5 mM, and 87.5 mM, respectively, for Preparations 45, 46, 20 47, and 48. To each of the four samples of 0.275 mL of the protein solution was added 1.1 mL of a crystallization buffer. The pH of each solution was adjusted to 7.6 with small quantities of 1 N HCl and 1 N NaOH. Each solution was then filtered through a 0.22 micron low protein binding 25 filter. To 1 mL of each solution was added 1 mL of a protamine sulfate solution prepared by dissolving 37.6 mg of protamine sulfate in 50 mL of water and then filtering through a 0.22 micron low protein binding filter. An

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amorphous precipitate formed in each case. Each preparation was stirred gently to complete mixing. The four preparations were allowed to stand undisturbed at 25°C for 23 hours.

5 Inspection under an optical microscope (1000x) revealed that in each case the amorphous precipitate had converted to a microcrystalline solid of uniform appearance, comprising single, rod-like crystals and that the mean crystal size of each preparation was different as tabulated:

| Preparation | [Citrate] (mM) | Crystal length (microns) |
|-------------|-------------------|-----------------------------|
| 45 | 0 | <3 |
| 46 | 5 | 3-6 |
| 47 | 15 | 5-10 |
| 48 | 35 | >10 |

10

Preparation 49

A dry powder of C10-BHI (60.7 mg) was dissolved in 1.50 mL of 0.1 N HCl. To this solution was added 600 microliters of a 15.3 mM solution of zinc chloride with stirring. To 0.70 mL of this solution was added 2 mL of an aqueous solvent composed of 50 mM TRIS, 10 mg/mL phenol, 30 mg/mL trisodium citrate dihydrate, and 31 mg/mL glycerol at pH 7.60. The pH was adjusted to 7.61 with small quantities of 1 N HCl and 1 N NaOH. This solution was then filtered through a 0.22 micron low protein binding filter. A second solution was prepared by dissolving 37.8 mg of protamine sulfate in 50 mL of water and then filtering through a 0.22 micron low protein binding filter. A 2.5 mL volume of the C10-BHI mixture solution was combined with 2.5 mL of the protamine sulfate solution. An amorphous precipitate formed. This suspension was stirred gently to complete mixing. The preparation was allowed to stand undisturbed at a temperature of 25°C for 60 hours. Inspection under an

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optical microscope (1000x) revealed that the amorphous precipitate had converted to a microcrystalline solid of uniform appearance, comprising small rod-like crystals with an estimated approximate mean particle size of 2 microns.

5

Preparation 50

A dry powder of C6-BHI (39.2 mg) was dissolved in 1000 parts by volume of 0.1 N HCl. To this solution was added 400 microliters of a 15.3 mM solution of zinc chloride with stirring. To this solution was added 4 mL an aqueous solvent comprised of 5 mg/mL dibasic sodium phosphate anhydrous, 25 mM trisodium citrate, 1.6 mg/mL phenol, 4 mg/mL m-cresol and 40 mg/mL glycerol at pH 7.6. The pH was adjusted to 7.60 with small quantities of 1 N HCl and 1 N NaOH. This solution was then filtered through a 0.22 micron low protein binding filter. A second solution was prepared by dissolving 37.3 mg of protamine sulfate in 50 mL of water and then filtering through a 0.22 micron low protein binding filter. A 5 mL volume of the C6-BHI solution was combined with 5 mL of the protamine sulfate solution. An amorphous precipitate formed. This suspension was stirred gently to complete mixing. The preparation was allowed to stand undisturbed at a temperature of 25°C for 47 hours. Inspection under an optical microscope (1000x) revealed that the amorphous precipitate had converted to a microcrystalline solid of uniform appearance, comprising single, rod-like crystals possessing an approximate mean length of 2 microns.

30

Preparations 51 - 54

The procedure of either Preparation 1 or Preparation 19 was followed as indicated in the table below to prepare 75% C8-BHI co-crystals, except that the

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crystallization buffer was prepared so that the final citrate and sodium chloride concentrations in the four samples were as follows:

| Preparation # | Process used | Final [citrate] (mM) | Final [NaCl] (mM) | VMSED (microns) | S.D. (microns) |
|---------------|----------------|----------------------|-------------------|-----------------|----------------|
| 51 | Preparation 19 | 10 | 0 | 9.243 | 2.46 |
| 52 | Preparation 19 | 10 | 50 | 5.314 | 1.32 |
| 53 | Preparation 19 | 2 | 20 | 6.67 | 1.69 |
| 54 | Preparation 1 | 10 | 0 | 7.812 | 1.77 |

5

After 24 hours, the amorphous precipitate had converted to rod-shaped crystalline material in each case. The VMSED and S.D. for each preparation are given in the table above.

10

Preparation 55

Formulation of Microcrystals

The microcrystals prepared according to any of Preparations 1 - 51 are separated from the mother liquor and 15 are recovered by conventional solid/liquid separation methods. The recovered microcrystals are then suspended in a solution consisting of 2 mg/mL sodium phosphate dibasic, 1.6 mg/mL m-cresol, 0.65 mg/ml phenol, and 16 mg/ml glycerol, pH 6.8, so that the final concentration of insulin 20 activity is about 100 U/mL.

Preparation 56

Formulation of Microcrystals

The microcrystals prepared according to any of 25 Preparations 1 - 51 are separated from the mother liquor and are recovered by conventional solid/liquid separation methods. The recovered microcrystals are then suspended in

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a solution consisting of 0.65 mg/ml phenol in water so that the final concentration of insulin activity is about 100 U/mL. A pH adjustment to approximately 6.8 is performed with 1 N HCl and 1 N NaOH.

5

Preparations 57 - 83

All preparations produced co-crystals of 75% C8-BHI. For all but three preparations (57, 62, and 63), the procedure of Preparation 19 was essentially followed, except that the crystallization buffer was adjusted as needed to achieve the final citrate and sodium chloride concentrations indicated. The scale was 32 - 50 mL. The pH at which dissolution of the proteins was carried out is indicated in the table. These preparations provide further support for the conclusions drawn above, and additionally, reveal that

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VMSED is inversely related to the citrate concentration at least in the range of 0 to 10 mM.

| Preparation No. | Citrate (mM) | NaCl (mM) | dissolution pH | VMSED (microns) | S.D. (micron s) |
|-----------------|--------------|-----------|----------------|-----------------|-----------------|
| 57 | 10 | 0 | 1.2 | 7.87 | 1.62 |
| 58 | 10 | 0 | 2.1 | 8.88 | 2.02 |
| 59 | 0 | 0 | 2.1 | 8.08 | 2.21 |
| 60 | 0 | 50 | 2.1 | 5.83 | 1.4 |
| 61 | 10 | 50 | 2.1 | 5.42 | 1.25 |
| 62 | 10 | 50 | 1.2 | 5.17 | 1.21 |
| 63 | 10 | 50 | 2.5 | 6.52 | 1.94 |
| 64 | 10 | 0 | 2.1 | 8.59 | 2.03 |
| 65 | 10 | 15 | 2.1 | 6.66 | 1.49 |
| 66 | 10 | 30 | 2.1 | 5.95 | 1.39 |
| 67 | 10 | 50 | 2.1 | 5.42 | 1.55 |
| 68 | 0 | 0 | 2.1 | 7.81 | 2.25 |
| 69 | 0 | 15 | 2.1 | 6.65 | 1.76 |
| 70 | 0 | 30 | 2.1 | 6.32 | 1.79 |
| 71 | 0 | 50 | 2.1 | Amorphous | |
| 72 | 4 | 0 | 2.1 | 7.60 | 2.22 |
| 73 | 4 | 15 | 2.1 | 6.43 | 1.57 |
| 74 | 4 | 30 | 2.1 | 6.01 | 1.47 |
| 75 | 4 | 50 | 2.1 | 5.89 | 1.34 |
| 76 | 0 | 20 | 2.2 | 5.94 | 1.85 |
| 77 | 1 | 20 | 2.2 | 6.27 | 1.94 |
| 78 | 2 | 20 | 2.2 | 6.29 | 1.84 |
| 79 | 4 | 20 | 2.2 | 6.36 | 1.75 |
| 80 | 0 | 50 | 2.2 | 5.02 | 1.78 |
| 81 | 1 | 50 | 2.2 | 6.52 | 2.07 |
| 82 | 2 | 50 | 2.2 | 6.55 | 2.17 |
| 83 | 4 | 50 | 2.2 | 6.31 | 1.88 |

5

Preparations 84 - 89

Effect of Zinc

All preparations produced co-crystals of 75% C8-BHI. For all preparations the procedure of Preparation 19 was essentially followed with the final citrate concentration of 10 mM and the final sodium chloride concentration of 50 mM. The scale was 20 mL. The pH at

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which dissolution of the proteins was carried out was 2.5. Zinc was added as indicated in the table. The zinc concentration is expressed as a weight proportion with respect to the total protein (insulin plus insulin derivative). Zinc at greater than 0.1% is required, and between 0.5% and 5.8%, zinc concentration no consistent effect on VMSED.

| Preparation No. | Zinc (%) | VMSED (microns) | S.D. (microns) |
|-----------------|----------|------------------------|----------------|
| 84 | 0.1 | Amorphous | - |
| 85 | 0.5 | 5.40 | 1.42 |
| 86 | 0.8 | 5.99 | 1.67 |
| 87 | 1.6 | 6.63 | 1.76 |
| 88 | 3.0 | 6.89 | 1.90 |
| 89 | 5.8 | 5.87 (hazy, pH 7.8) | 1.85 |

10

Preparation 90

Preparation of 85% Nε-octanoyl-LysB29 Human Insulin and Human Insulin cocrystallized with protamine and Zn

A dry lyophilized powder of Nε-octanoyl-LysB29 Human Insulin (1678.5 mg) was dissolved in 42.5 mL of 0.1 N HCl. A dry powder of human insulin-Zn crystals (286 mg) was dissolved in 7.5 mL of 0.1 N HCl. 7.5 mL of the later solution was mixed with Nε-octanoyl-LysB29 Human Insulin solution to produce a mixture of Nε-octanoyl-LysB29 Human Insulin and human insulin in approximate weight ratio of 75:25. This solution was stirred to mix. To this solution was added 1.38 grams of a 10 mg/mL solution of Zn. To this solution was added 200 mL of an aqueous solution composed of 9.5 mg/mL disodium phosphate heptahydrate, 0.375 M NaCl, 4 mg/mL m-cresol, 1.65 mg/mL phenol, and 16 mg/mL glycerol at pH 7.63. The pH was adjusted to 7.6 with small quantities of 5N HCl and 5N NaOH. This solution was then filtered

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through a 0.2 micron low protein binding filter. A second solution was prepared by dissolving 262.9 mg of a dry powder of protamine sulfate in 305.69 grams of water and then filtered through a 0.2 micron low protein binding filter.

5 262.84 grams of solution of a mixture of N^ε-octanoyl-LysB29 human insulin and human insulin was mixed with 259.6 grams of protamine sulfate solution. A white precipitate formed. This suspension was stirred gently to complete mixing. The preparation was allowed to stand undisturbed for 24 hours at
10 32°C. Inspection under an optical microscope revealed the presence of microcrystalline solid. Measurement of particle size distribution by Coulter technique revealed a mean particle diameter of 2.5 microns. The mother liquor of this preparation was exchanged with an aqueous solution composed of 0.04 mg/mL phenol, 0.11 mg/mL m-cresol and 0.3 mg/mL of Disodium phosphate heptahydrate and 24 mg/mL of glycerin by
15 the following procedure. 450 mL of supernatant was removed by aspiration from a well settled suspension without disturbing the solid phase and replaced with the aqueous solution described above. Analytical characterization by HPLC revealed a composition of 85.2% C8-BHI and 14.8% BHI. For dog intrabronchial instillation experiments, these formulations were diluted to the required concentration by appropriate dilution with the aqueous solution used for
20 exchange described above. The microcrystals in this preparation had a mean particle diameter of 2.5 microns by Coulter multisizing. This preparation was used for
25 intrabronchial instillation in dogs.

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Preparation 91

Isolation of 85% cocrystal formulation (from preparation 90 above) in powder form (Approximately 1 gram scale)

5 Approximately 400 mL of the crystalline suspension from Preparation 90 was filtered through a 0.22 micron filtration apparatus by application of vacuum. The solid was washed with approximately 5 mL of absolute ethanol and air-dried by the application vacuum to the filtration apparatus. The 10 agglomerated powder was collected using a clean spatula.

Preparation 92

In vitro dissolution properties of 85% cocrystal formulation (from preparation 90 and preparation 91 above)

15 The *in vitro* dissolution rate of the microcrystalline suspension from preparation 90 was measured in phosphate buffered saline (PBS) at pH 7.4 and a temperature of 25 degrees centigrade. The PBS buffer contained 1 mg/mL of 20 bovine serum albumin to minimize adsorption loss of insulins. A volume of suspension that contains 1.8 mg of total insulin is suspended in 200 mL of buffer solution and stirred at a constant rate of 180 rpm. At regular interval aliquots of the solution was filtered through a 0.2 micron 25 low protein binding filter and assayed to determine total dissolved insulins. Unfiltered control samples were also analyzed to determine total available insulins. Based on this assay, preparation 90 required approximately six hours for complete dissolution. In comparison, NPH crystals 30 dissolved in about 2 minutes. Furthermore, as preparation 90 dissolved, it released a constant ratio of C8-BHI to BHI, confirming the co-crystalline nature of preparation 90. The isolated powder (preparation 91) retained the slow 35 dissolution properties of preparation 90 suggesting that the process of isolating the powder and drying did not affect

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the dissolution properties. For the powder, approximately 50% dissolution occurred in about three hours. During this period, as it dissolved, the powder released a constant ratio of about 85% C8-BHI and 15% BHI. This property of 5 homogeneous dissolution further confirms that the process of filtration and air-drying does not alter the fundamental nature of the microcrystals.

These in-vitro dissolution data suggest that these microcrystals with mean particle diameter of about 2.5 10 microns dissolve very slowly compared to NPH, despite their increased surface area. These data support the potential of Preparation 90 to serve as a sustained release formulation. These data further support that the process of isolation and 15 drying do not alter the unique dissolution characteristics of the suspension.

Example 1

Two types of crystalline insulin were studied, 100% C8-BHI crystals and 75% C8-BHI:25% BHI co-crystals. 20 For both of these, intratracheally instilled compounds produced blood levels of immunoreactive insulin that were higher for a longer sustained period than seen in previous studies conducted in rats with regular insulin and comparable to results with subcutaneously delivered NPH- 25 human insulin. Interestingly, when lung lavage fluid was examined at 4 and 8 hours after lung instillation, insulin crystals were observed primarily free and intact in the lung lavage fluid with only relatively few crystals seen within alveolar macrophages. The expectation was that most of the 30 crystalline material would have been taken up by the alveolar macrophages and digested since the literature shows that particle ingestion by macrophages in the lung is largely complete within a few hours. This surprising

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finding suggests that if crystalline insulin is insoluble enough not to dissolve too quickly in lung fluids it will remain available for slow dissolution and absorption into the blood. Therefore, lung delivery of these two 5 crystalline insulins may be a non-invasive approach for sustained release of insulin to provide basal control of glucose.

Glucose levels were measured in these rat experiments and they were depressed commensurate with the 10 absorbed levels of insulin into blood. These experiments show that lung delivery is a feasible method for delivering insoluble insulin crystals to the lung for sustained release of insulin into the blood. Aerosol inhalation will be the means used in clinical use to delivery the crystalline 15 insulin to the lungs of patients to obviate the need for injections and improve patient compliance.

Example 2

Twelve male F344 rats/group were used in this 20 study. The dose groups were as follows:

Group 01 Intratracheal Instillation of 1 mg/kg of 100% C8-BHI, prepared according to Preparation 28, above.

25 Group 02 Intratracheal Instillation of 1 mg/kg of 75% C8-BHI:25% BHI, prepared according to Preparation 29, above.

Group 03 Subcutaneous Administration of 1 mg/kg of 75% C8-BHI:25% BHI, prepared according to Preparation 29, above.

30 Group 04 Subcutaneous Administration of 1 mg/kg of NPH insulin

Blood samples were collected at 0 (pre), 0.5, 1, 4, 8, 16, and 24 hours after dosing. These blood samples were centrifuged and the serum was collected to determine 35 blood levels of the test article and glucose concentrations.

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The results of the glucose determinations are presented in Figure 1. These preparations instilled into rat trachea provided lowering of blood glucose, sustained release comparable with subcutaneous delivery of NPH-human insulin, and surprisingly high activity.

These data create a reasonable expectation that these crystals and co-crystals can be administered by inhalation, that particles having properties known to make them likely to deposit in the lungs of a patient would be deposited in the lung of a human patient in need of insulin to control blood glucose, and that these crystals or co-crystals would dissolve within the lung, and the insulin activity would be absorbed into the patient's blood from the deposited particles.

15

Example 3

Physical Stability and Resuspendability Testing

This study evaluated physical stability under physical stress conditions for four different 75% C8-BHI co-crystal formulations in 3.0-mL Cartridges. Preparations 51-54 were filled into 3.0 mL cartridges and placed on an accelerated physical stability test. Forty-four cartridges were filled for each Preparation.

Thirty-eight cartridges of each Preparation were placed in an insulin agitator system at a constant temperature of 37°C for 14 days. During this period the materials were agitated by rotation at 30 rpm, 4 hours per day. The cartridges were inspected for fibrillation (aggregation or agglomeration) and physical changes were noted on days 0, 2, 5, 7, 9, 11, and 14. A set of control cartridges (6 for each Preparation) were stored at 5°C (vertical cap side up) without agitation (except for resuspension) and were inspected every 7 days.

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Cartridges were failed for the appearance of any of the following conditions, as determined by visual examination by trained personnel: conversion from a milky white, uniform suspension with slow sedimentation to one exhibiting discrete particles having more rapid sedimentation; film on cartridge walls; frost; clumping (large aggregates); loss of resuspension; or any combination of the above reasons.

After 14 days of accelerated physical stability testing, Preparations 51 and 54 had roughly the same number of failed cartridges. Preparation 53 exhibited the least number, and Preparation 52 had only slightly more failures than Preparation 53, but less than either Preparations 52 or 55.

15

Example 4

Glucose Response to Powders of 85% C8 Insulin:15%BHI Co-Crystals Insufflated into the Lungs of F344 Rats.

20 To determine the effects of airborne powders delivered to the lung, an experiment was carried out in which 85% C8 insulin powders (preparations 90-92) were blown into the lungs of rats using an insufflation method. A dose of 2 mg/kg was used.

25 Fasted male Fischer 344 rats (200-250 gm) were briefly anesthetized with isoflurane and intratracheally intubated. Pre-weighed aliquots of powder were prepared with inhalation grade lactose employing geometric dilution to assure uniform mixing of the insulin crystals at a concentration of 5% by 30 weight with the carrier lactose. 10 mg of powder was filled into size 00 capsules that were then loaded into a Penn Century insufflation device. The Penn Century device was introduced into the intratracheal cannula and the powder blown into the lungs by rapidly expelling 3 ml of air from a

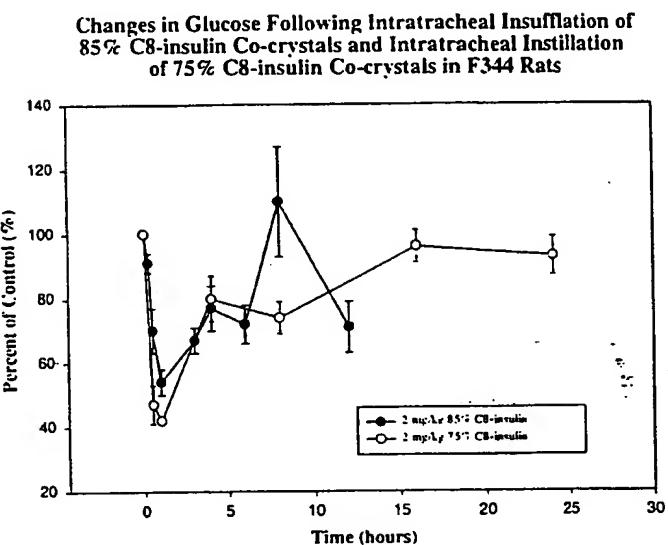
-105-

hand-operated syringe. Dosing was synchronized with the animal's inspiration. Blood samples were taken at 0 (pre-dose), and 0.25, 0.5, 1, 3, 4, 6, 8, and 12 hours after dosing for glucose measurements.

5 The figure below shows the glucose response (mean \pm SE) following insufflation of powders of 85% C8insulin:15% BHI crystals into rat lungs. The data point at 8 hrs is considered as an outlier because for some unknown reason there were two rats in this group that had glucose levels 10 that were more than 50% of their baseline levels. Ignoring this data point, the glucose responses show an extended time-action profile quite similar to the data obtained following instillation of 75% C8 Insulin:25% BHI crystal suspensions obtained in the previous experiment above. These 15 data show both the airborne powders introduced into the lung and the liquid suspensions produce similar responses. Previous rat experiments had shown qualitatively similar glucose response following instillation of the various types of crystalline insulins. These results, in aggregate, 20 suggest that the results from instillation studies should be good predictors of results from airborne powders introduced into the lung. As shown in this experiment one crystalline insulin showed extended time action of glucose response following introduction airborne powder into the lung and

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similar behavior is expected for the other crystalline insulins when introduced into the lung as airborne powders.



5

Example 5

Comparison of 3 Crystalline Formulations of Insulin Delivered Intrabronchially to Beagle Dogs

The present study was designed to compare the kinetics following subcutaneous (sc) administration of NPH crystals to the kinetics following intrabronchial (IB) administration of NPH crystals, MicroUltralente crystals, and 85% C8-insulin co-crystals.

The live phase of the study was performed at Lilly's Toxicology Research Laboratories. Two male and three female adult beagle dogs were used in this study. Weights ranged from 7.1 to 14.6 kg at the start of the study.

The test articles used were 85% C8-insulin co-crystals, MicroUltralente crystals, and NPH crystals. Each test article was specifically formulated for pulmonary administration using modified crystallization conditions to

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produce 2-3 micrometer particles. Specific vehicles were used for each crystalline insulin formulation as shown in Table 1.

5

Table 1. Crystalline insulin formulations and their associated vehicles.

| Compound | Vehicle |
|----------------------|--------------------------------------------------------------------------------------------------|
| 85% C8- insulin | Aqueous solution containing 0.7 mg/ml phenol, 1.6 mg/ml metacresol, 0.3 mg/ml phosphate |
| MicroUltra- lente | Water |
| NPH | Water, glycerin, and trace amounts of phenol and metacresol |

Anesthetized, fasted animals were dosed with 85% C8-insulin co-crystals, MicroUltralente crystals, and NPH crystals via pulmonary or subcutaneous routes of administration. Pulmonary doses, as shown in Table 2, were delivered intrabronchially via a fiberoptic bronchoscope with instillation volumes of 0.25 ml/kg. For comparative purposes, each dog was dosed with 0.75 U/kg of NPH crystals subcutaneously. At least 7 days elapsed between each treatment. Blood samples were collected pre-treatment, and at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 16, and 24 hours after administration. For the subcutaneous and pulmonary doses of NPH, blood samples were collected at pre-treatment, and at 0.5, 1, 2, 4, 6, 8, 10, 12, 16, and 24 hours after

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dosing. Serum obtained from these samples was analyzed for glucose concentrations.

Table 2. Delivered Doses

| Compound | Dose (Units/kg) | Route |
|-------------------------------|--------------------|--------------------|
| NPH crystals | 0.75 | Subcutane ous |
| NPH crystals | 1.5 | Intrabron chial |
| 85% C8-insulin co-crystals | 1.5 | Intrabron chial |
| MicroUltraLente | 0.75 | Intrabron chial |

5

Results and Discussion

Intrabronchial instillation of all crystalline insulin formulations produced a significant suppression of serum glucose levels (Table 3, Figure 1). The average blood 10 glucose level reached an initial low point at 2 hours for all formulations.

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Table 3.
Blood glucose (percent of baseline) after intrabronchial or
subcutaneous administration of crystalline insulin
formulations in Beagle dogs.

| Time (hrs) | C8-insulin | | Micro-ultralente | | sc | | IB | |
|---------------|----------------------|--------------|---------------------|--------------|--------------|--------------|--------------|--------------|
| | co-crystals (n=5) | Std Error | ultralente (n=3) | Std Error | NPH (n=5) | Std Error | NPH (n=5) | Std Error |
| 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 0.5 | 89 | 2 | 96 | 8 | 91 | 5 | 80 | 10 |
| 1 | 50 | 7 | 72 | 9 | 56 | 5 | 45 | 12 |
| 2 | 40 | 4 | 32 | 6 | 40 | 4 | 37 | 5 |
| 3 | 41 | 8 | 39 | 4 | | | | |
| 4 | 50 | 7 | 44 | 6 | 47 | 5 | 46 | 10 |
| 5 | 34 | 6 | 31 | 5 | | | | |
| 6 | 46 | 13 | 44 | 5 | 51 | 7 | 35 | 5 |
| 7 | 46 | 14 | 38 | 3 | | | | |
| 8 | 45 | 14 | 36 | 6 | 50 | 8 | 33 | 5 |
| 9 | 46 | 16 | 40 | 4 | | | | |
| 10 | 63 | 17 | 49 | 4 | 77 | 7 | 56 | 7 |
| 12 | 71 | 21 | 80 | 10 | 93 | 3 | 95 | 17 |
| 16 | 89 | 13 | 93 | 4 | 102 | 7 | 111 | 6 |
| 24 | 102 | 7 | 98 | 0 | 92 | 4 | 102 | 4 |

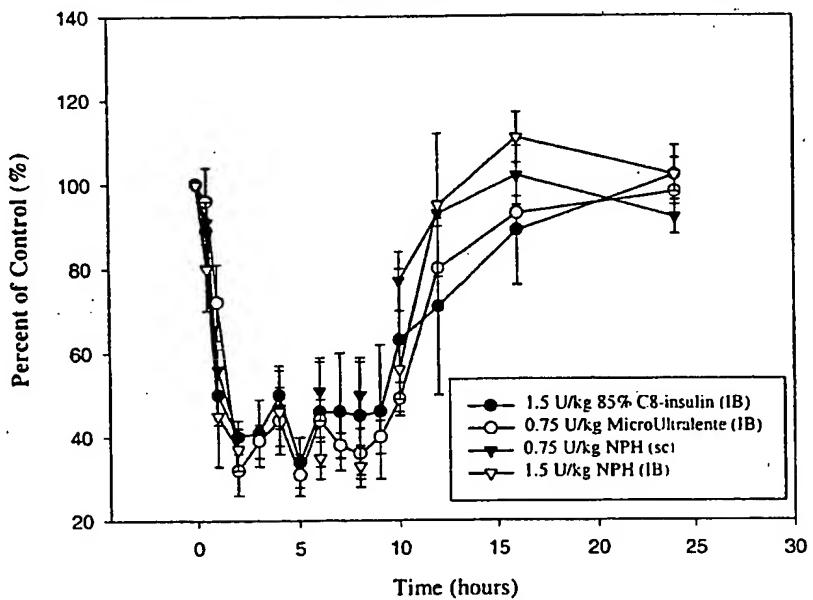
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Glucose levels remained markedly depressed up to approximately 10 hours following intrabronchial or subcutaneous administration for all formulations. The time-action for sustained release was similar for intrabronchial instillation of all formulations and was at least as long as subcutaneous administration of NPH in all cases. Two dogs following intrabronchial administration of NPH and 85% C8-insulin co-crystals experienced critically low glucose levels between 7 and 10 hours post-dose and were rescued

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using 50% dextrose administered either orally or intravenously. These episodes artificially increased serum glucose levels in the rescued animals and therefore shortened potential extended release action and the overall 5 mean of the group.

Changes in Serum Glucose Levels (mean +/- SE) Following Subcutaneous Administration of NPH or Intrabronchial Administration of NPH, MicroUltralente, and 85% C8-insulin Co-crystals



10

Example 6

Aerosol Characterization for 3 Crystalline Formulations of Insulin

Three dry powder formulations of crystalline insulin 15 were aerosolized using a Wright Dust Feed (WDF) generator operated at 10 LPM. The aerosol generated by the WDF either

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passed through a cyclone designed to remove large particles before entering a 12-L head-dome exposure system or passed directly from the WDF into a head-dome. The mass median aerodynamic diameter (MMAD) was determined by using a Sierra 5 Model 218K Cascade Impactor fitted with Gelman Type A/E glass fiber filters. Airflow through the Cascade Impactor was 3 L/minute with sample times ranging from 5 to 50 minutes. Aerosol concentration was determined by collecting a gravimetric sample during the exposure.

10

Results

Particle size and gravimetric data for the 3 crystalline insulins are shown in Table 1.

15

Table 1. Aerosol Characterization

| Test Articles | MMEDD | GSD | Mean (\pm SE) Aerosol Concentration (mg/L) |
|----------------------------------------|-------|------|--------------------------------------------------------|
| 85% C8-insulin | | | |
| w/ cyclone | 1.15 | 3.98 | NA |
| w/o cyclone | 1.30 | 4.03 | 0.069 |
| 10% MicroUltralente/90% Lactose | | | |
| w/ cyclone | 2.74 | 1.61 | 0.280 |
| w/o cyclone | 4.07 | 1.97 | 0.104 |
| Spray-dried MicroUltralente | | | |
| w/ cyclone | 1.79 | 2.90 | 0.088 |
| w/o cyclone | 2.31 | 3.04 | 0.040 |

These data show that highly respirable particle size distribution result upon aerosol generation of these crystalline insulins.

20

The data from Examples 1-5 are convincing that instillation studies are predictive for effects of inhaled dry powders. Studies have been conducted in beagle dogs to compare glucose responses following a) inhalation of

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solution aerosols of regular insulin, b) inhalation of powders of regular insulin, and c) instillation of a solution of regular insulin. The accompanying figure shows that the time-action profile of the glucose response is 5 rapid in all cases and the results are similar for all 3 types of dose administration. The time to minimum glucose levels is less than 2 hours in all cases, and responses are returning to near baseline within 4-5 hours. This profile is also similar to that achieved in humans following 10 inhalation of regular insulin either as liquid aerosols (Laube BL, Georgopoulos, Adams GK. 1992. *Aerosolized* insulin delivered through the lungs is effective in normalizing plasma glucose levels in non-insulin dependent diabetes. *J. Biopharm. Sci* 3 (1992) 163-169) or powders 15 (Patton J, Bukar J., and Najarajan S. 1999. *Inhaled* insulin. *Adv. Drug Delivery Rev.* 35: 235-247). These results lend confidence to predicting similar results in inhalation studies to those achieved with instillation studies. The similar glucose response in rats following 20 administration of intratracheally instilled suspensions of C8 co-crystals compared to insufflation of powders of the same C8 co-crystals also directly supports this view.

The invention has been described with reference to 25 various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. All publications and patent applications in this specification are indicative of the 30 level of ordinary skill in the art to which this invention pertains.

We claim:

1. Crystals having a uni-modal, symmetric particle distribution, comprising:

a) derivatized insulin of the formula B29-NE-X-

5 human insulin, wherein X is selected from the group consisting of butyryl, pentanoyl, hexanoyl, heptanoyl, octanoyl, nonanyl, and decanoyl;

b) zinc;

10 c) a phenolic preservative; and

d) protamine;

characterized in that the volume mean spherical equivalent diameter is from 1 microns to 3 microns.

15 2. Crystals according to Claim 1 further comprising insulin, wherein the proportion of derivatized insulin relative to total protein is at least 50%.

3. Crystals according to Claim 1 or Claim 2, wherein the derivatized protein is B29-NE-octanoyl-human insulin.

20 4. Crystals according to either Claim 2 or Claim 3, wherein the proportion of derivatized insulin is 75%.

5. Crystals according to any one of Claims 1 - 4, wherein zinc is present at about 0.3 mole to about 0.7 mole 25 per mole of total protein.

6. Crystals according to any one of Claims 1 - 5, wherein the phenolic preservative is selected from the group consisting of phenol, m-cresol, and a mixture of phenol and m-cresol, and wherein the phenolic preservative is present 30 at least in a ratio of 0.5 mole per mole of total protein.

7. Crystals according to any one of Claims 1 - 6, wherein protamine is present at about 0.15 mg to about 0.5 mg per 3.5 mg of total protein.

8. A pharmaceutical composition for 5 administration by inhalation by mouth, comprising crystals according to one of Claims 1- 7, and additionally a carrier, an absorption enhancing compound, an excipient, or a solvent.

9. The pharmaceutical composition of Claim 8, 10 further comprising insulin, an insulin analog, or derivatives or pharmaceutically acceptable salts thereof.

10. Use of the crystals according to any one of Claims 1 - 7 to prepare a medicament for the treatment of diabetes.

15 11. A process for preparing crystals according to any one of Claims 1 - 7, comprising preparing a suspension having neutral pH, in the absence of citrate, by carrying out steps a) - f) in any order, provided that step f) follows step a), and provided that if step f) precedes step 20 e), then steps b) and c) precede step f):

- a) dissolving a derivatized insulin in an aqueous solvent at acidic pH;
- b) adding a phenolic preservative;
- c) adding zinc;
- 25 d) adding chloride anion to a final concentration of from about 100 mM to about 150 mM chloride anion above that introduced by pH adjustment;
- e) adding protamine;
- 30 f) adjusting to a neutral pH; and then holding the temperature of the neutral pH suspension between about 25°C and about 37°C for between 12 hours and about 96 hours.

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12. The process of Claim 11, wherein insulin is also dissolved and wherein the proportion of derivatized insulin relative to total protein is at least 50%.

13. The process of either Claim 11 or Claim 12,
5. wherein the derivatized insulin is B29-N^E-octanoyl-human insulin, chloride anion is added to a final concentration of between about 120 and 150 mM above that introduced by pH adjustment, and the temperature is about 30°C.

14. The process of Claim 13, wherein the
10 derivatized insulin is B29-N^E-octanoyl-human insulin and the proportion of derivatized insulin is 75% of total protein, chloride anion is added to a final concentration of between 120 and 150 mM above that introduced by pH adjustment, and the temperature is about 30°C.

15. 15. A method of treating diabetes comprising parenterally administering crystals according to any one of Claims 1 - 7.

16. A method of treating diabetes comprising administering crystals according to any one of Claims 1 - 7
20 by inhalation via the mouth.

17. A process for preparing crystals, comprising preparing a suspension having neutral pH, in the absence of citrate, by carrying out steps a) - g) in any order, provided that step g) follows step a), and provided that if
25 step g) precedes step f), then steps b) and c) precede step g):

a) dissolving a derivatized insulin in an aqueous solvent at acidic pH;
b) adding a phenolic preservative;
30 c) adding zinc;
d) adding chloride anion to a final concentration of from about 15 mM to about

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150 mM chloride anion above that introduced by pH adjustment;

5 e) adding citrate to a concentration of from 1 mM to 10 mM;

f) adding protamine;

g) adjusting to a neutral pH;

and then holding the temperature of the neutral pH suspension between about 20°C and about 37°C for between 12 hours and about 96 hours.

10 18. The process of Claim 17, wherein insulin is also dissolved and wherein the proportion of derivatized insulin relative to total protein is at least 50%.

15 19. The process of Claim 18, wherein the proportion of derivatized insulin is 75%.

20 20. The process of Claim 19, wherein the derivatized protein is B29-NE-octanoyl-human insulin.

21. The process of any one of Claims 17 - 20, wherein zinc is added to a concentration between 0.3 mole and 0.7 mole per mole of total protein, the phenolic preservative is selected from the group consisting of phenol, m-cresol, and a mixture of phenol and m-cresol and is added to a concentration to provide at least a ratio of 0.5 mole of phenolic preservative per mole of total protein, and protamine is added to a concentration between 0.15 mg to 25 0.5 mg per 3.5 mg of total protein.

22. Crystals produced by any one of Claims 17 - 21.

23. A pharmaceutical composition comprising the crystals of Claim 22, and additionally a carrier, an absorption enhancing compound, an excipient, or a solvent.

30 24. The pharmaceutical composition of Claim 23, further comprising insulin, an insulin analog, or derivatives or pharmaceutically acceptable salts thereof.

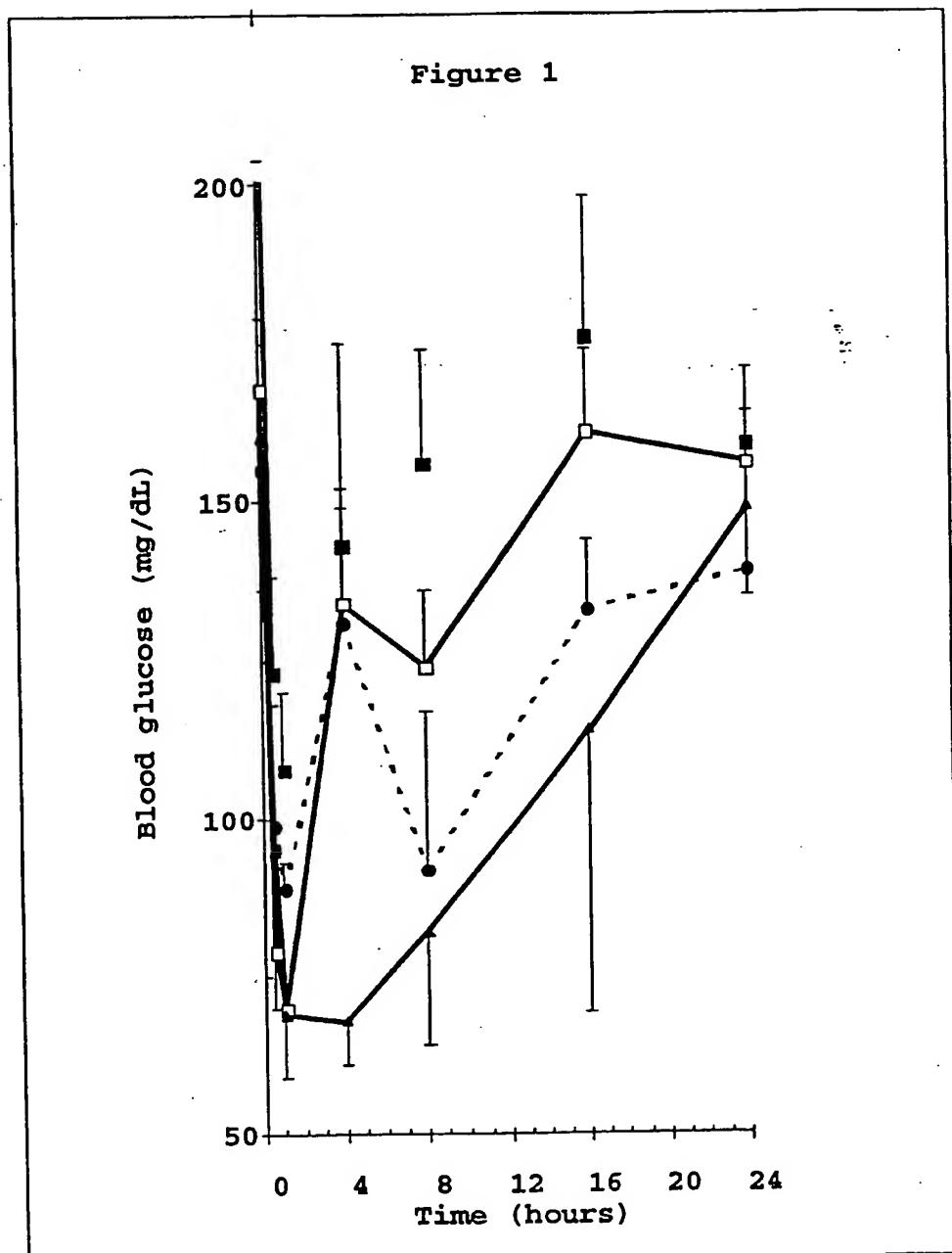
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25. Use of the crystals of Claim 22 to prepare a medicament for the treatment of diabetes.

26. A method of treating diabetes comprising parenterally administering the crystals of Claim 22.

1/1

FIG. 1



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/08723

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C07K14/62 A61K38/28 A61K9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS

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INTERNATIONAL SEARCH REPORT

International Application No

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